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VOLUME LXXVIII

FEBRUARY TO JUNE, 1940

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Mass., between June 1 and October 1 and to the Biological Laboratories, Divinity Avenue, Cambridge, Mass., during the remainder of the year.

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Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

LANCASTER PRESS, INC., LANCASTER, PA.

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Vol. LXXVIII, No. 1 February, 1940

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

POSTURAL REACTIONS OF INSECT ANTENNAE

ERNST WOLF

(*From the Biological Laboratories, Harvard University, Cambridge, Massachusetts*)

I

A bee held in a fixed position can respond to a motion of a visual pattern made up of stripes of equal width which is moved sidewise. The response is a definite movement of the antennae. The antennae assume with the onset of the shift of the pattern a definite position which is kept as long as the motion of the pattern continues; with cessation of the stripe motion the antennae move freely and independently. If, for instance, the pattern be moved from left to right, the left antenna will point at an angle of 90° to the bee's axis while the right antenna points straight forward. On reversing the motion of the pattern, the antennae take the reverse position (Wolf and Zerrahn-Wolf, 1935).

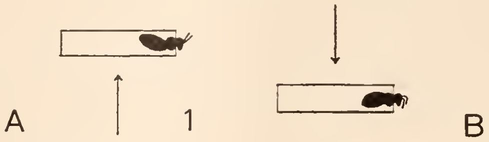
From earlier tests of visual acuity (Hecht and Wolf, 1929), intensity discrimination (Wolf, 1933*a* and *b*) and flicker (Wolf, 1933*c*) with freely creeping bees, it is known that a bee responds to a sudden displacement of a striped pattern which is moved underneath its creeping compartment, by a change of its course in a direction *against* the stripe motion. It therefore seems likely that the posture of the antennae recognizable with the bee held in a fixed position while viewing the moving stripes, is an expression of the bee's tendency to adjust its body posture in relation to the visual stimulus—just as a freely moving bee does by its turn against the stripe motion.

Postural responses of the antennae like those evidenced on stimulation by movement of a visual pattern can also be recognized under stimulation by gravity and centrifugal force.

II

If a bee is mounted in a small glass tube in such a way that the head sticks through a thin rubber membrane stretched across the end of the tube, while the thorax and abdomen are within the tube, the animal can easily be exposed to gravitational and centrifugal stimulation and the postures of the antennae observed. The head end of the tube can be enclosed in a larger glass vessel so as to avoid the influence of gross air currents during motion, while the open abdominal end provides ample air for respiration. If properly fed, bees can be kept mounted in this way for days, and may be used repeatedly for experimentation. All responses described here are observed under conditions avoiding visual stimulation.

Vertical movements: If the animal is being lifted upward rapidly, both antennae swing upward and are kept rigid in this position until the movement ceases; thereafter the antennae move freely without any particular coördination of motion being noticeable (Fig. 1, *A*). During a downward movement both antennae are bent at a sharp angle, at the joint between the base and the flagellum, the tips of the antennae pointing almost straight downward (Fig. 1, *B*). During this motion both antennae also stay rigid and begin to move freely again when the downward motion ends. In repeating the up or down motion the antennae take their respective positions with great precision.



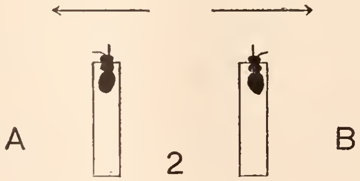


Fig. 1. Postures of the antennae of a bee held in a glass tube, when suddenly lifted vertically upward (*A*), and (*B*) when giving it a sudden downward motion.

Fig. 2. Postures of the antennae of a bee (seen from above) when moved horizontally with a motion perpendicular to the bee's body axis. Motion to the left (*A*) and to the right (*B*).

Horizontal movements: When moving a bee enclosed in a glass tube horizontally so that the direction of motion is perpendicular to the body axis, the antennae take definite position in relation to the direction of motion. The leading antenna (left antenna, while moving to the left) points at an angle of 90° to the body axis, whereas the following antenna points straight forward (Fig. 2, *A*). On reversing the direction of motion the antennae take reverse positions, the right antenna being bent at 90° to the body axis and the left antenna extended straight (Fig. 2, *B*). That the antennae postures are not conditioned by visual stimuli is easily shown by the fact that the typical postures are induced by moving the bee against a uniform white background, or by making the tests in a darkroom with only a dim red light illuminating the bee.

If the bee is moved horizontally around in a large circle ($r = 1$ m.) the antenna toward the centre of the circle is bent at almost an angle of 90° while the outer antenna is extended straight (Fig. 3). Movements in the line of the bee's axis, however, do not elicit any definite position of the antennae; both can be held rigid at an angle of about 45° to the body axis or move freely and independently. The same holds for rectilinear vertical motion while the bee is facing straight upward or downward.

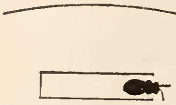


FIG. 3. Postures of the antennae of a bee (seen from above) when moved horizontally on a circular course, facing in the direction of motion.

III

For the study of postural responses of the antennae of the bee under centrifugal force the following procedure was used (Fig. 4). On a turntable of 42 cm. diameter two right-angle prisms are mounted, one in the centre of the disc and the other close to the periphery. Underneath the turntable directly below the peripheral prism is a flashlight. Through an Eastman filter No. 91 the light illuminates an insect mounted in its glass enclosure and held by clamps in radial position on the turntable. Through the prism at the centre of the disc the bee's head is observed against the dark red background. By using a telescope above the central prism, focused on the bee's head, every motion and change in position of the antennae can easily be observed.

When a bee is placed in the holder with the turntable in *horizontal* position, and is facing radially toward the periphery of the turntable, typical postural responses of the antennae appear as the disc is slowly rotated through an angle of about 90° per second. The antenna of the side of the body in the direction in which the disc is turned points at an angle of 90° to the axis of the bee, while the other antenna points straight forward (Fig. 5). This position is held as long as the disc is in motion. On stopping, temporary compensatory movements of the antennae into reverse positions can be observed. When the bee in its tube is held so that its head points toward the centre of the disc, the same

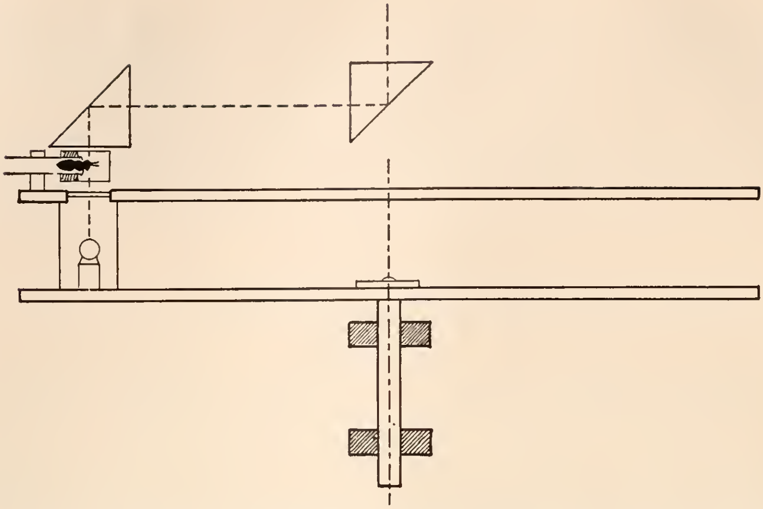


FIG. 4. Turntable for observation of postural responses of the antennae of the bee under the influence of centrifugal force; diagrammatic vertical section, through the axis of rotation, the holder for the insect, and the optical system.

antennal postures are noticed during rotation, i.e., the leading antenna bends at an angle of 90°, while the other antenna is straight.

With the turntable placed *vertically* the antennal postures show a dependence upon the bee's spatial position. A bee facing toward the centre of the disc while moving down will point downward with the leading antenna, while the other is straight. On arriving at the lowest point of the vertical circular course, both antennae are fully extended, almost parallel and straight. When moving upward again the leading antenna bends and reaches an angle of 90° to the body axis when the bee's axis has reached the horizontal. On moving further up so that in the extreme case the bee faces down vertically, both antennae are bent and retracted close to the head (Fig. 6, *A*). Passing the topmost point and then bringing the bee into the position from which the rotation was started, the original position of the antennae is again taken. When the bee is

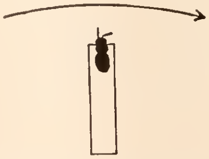


FIG. 5. Postures of the antennae of a bee during rotation when placed on a horizontal turntable facing radially toward the periphery.

facing toward the periphery of the turntable the leading antenna again points at an angle of 90° to the body axis and the other is straight, while the animal is either on its way downward or upward; whereas at the lowest point of the course both antennae are flexed and at the highest point both are fully extended (Fig. 6, *B*).

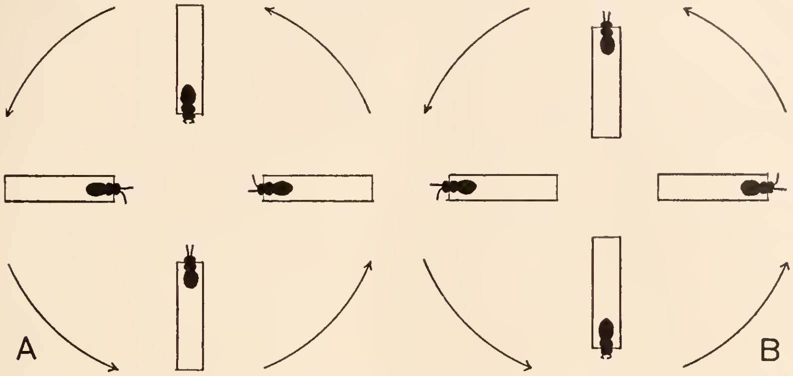


Fig. 6. Postures of the antennae of a bee during rotation on a turntable in vertical position: facing toward the center of the turntable (*A*), and toward the periphery (*B*).

Postural responses of the antennae are also exhibited when a bee in vertical position is rotated around its long body axis. The leading antenna points straight toward the side of the animal or is extended forward in the direction of rotation, whereas the following antenna is extended straight upward or is bent at the joint between base and flagellum, the tip then pointing also in the direction of rotation.

IV

On a turntable a free moving bee creeps generally against the direction of rotation, in a roughly circular course, if no visual stimuli are involved. The diameter of the bee's circular path depends upon the velocity of rotation, the diameter varying inversely with the speed of rotation. On a light background with dim red illumination the position of the bee's antennae can be followed in the darkroom. While the bee is moving freely—and when placed on the turntable it is almost continuously in motion—both antennae point at about an angle of 45° to the axis of the body. Only in case the bee has been resting in such a position that it faces radially toward the periphery or toward the centre of the turntable, can sudden motions of the antennae be noticed when the disc is set into rotation. As in the case when the bee is held in a fixed position, the antenna pointing in the direction of rotation swings out to an angle of 90° to the body axis, while the other antenna points straight forward. The rotation, however, at once initiates circus movements of the bee; thus the bee starts its circular course and both antennae take positions at an angle of about 45° to the body axis. It has to be assumed that if the initial course of the bee, at the moment when the rotation begins, runs in the direction of the antenna which points at an angle of 90°, the bee's motion *against* the rotation of the turntable can only occur when the bee is facing toward the centre of the turntable; whereas a movement *with* the rotation should occur when the bee is facing toward the periphery. That this is the case can easily be demonstrated by waiting for a moment when the bee is moving radially toward the periphery of the turntable before starting the rotation. The bee under these conditions swings in a path in the direction of rotation of the disc, but as soon as the bee has described a quarter of a circle the animal's position becomes such that the leading rôle of the antenna which initiated the turn in that direction is taken over by the other one; consequently the bee describes a circular motion to the opposite side and now describes a course *against* the rotation of the disc. When the motion of the bee is not too fast, the shift in position of the antennae can be observed. It should be stated, however, that since the animal has to be kept underneath a glass cover and since, furthermore, its position is only rarely truly radial the picture is easily obscured. Stereotactic responses and unbalanced onset of excitation can lead to an initial turning *against* the rotation or can through contact with the wall of the container prolong the course *with* the rotation.

V

In all the cases described thus far, attention was paid only to the postures of the antennae. With the bee sealed in a tube, the only freely movable appendages are the antennae. Leg movements, leg postures, and the posture of the abdomen are hardly recognizable since the legs and the abdomen are or can be in close contact with the wall of the glass tube in which the bee is held. There are, however, indications that the abdomen takes definite postures as a result of gravitational and other mechanical stimulation.

If a bee is resting on its legs, a slight pressure on one side of the abdomen will initiate at once a bending of the antenna to the side where the pressure is applied, whereas the other one is extended straight forward. On releasing the pressure the abdomen swings to the side from which the pressure came. The same is true in case of applying an air current from one side. If under such conditions the bee turns around its centre, it always tends to move in a small circle into such a position that it faces the air current. These responses are comparable to the homostrophic responses described in *Tenebrio* larvae (Crozier, 1924) and diplopods (Crozier and Moore, 1923).

Abdominal posture change is more clearly evident when during applied vertical motion the bee is held by the back of the thorax while the legs and the abdomen can be moved freely. During an upward motion the abdomen is then held straight or slightly bent upward at its tip, while during a downward motion it is strongly curved downward (as indicated in Fig. 1). Even during motions perpendicular to the bee's body axis while held in a tube, bending of the abdomen can be observed (Figs. 2 and 5), but these postural changes are never as clear and persistent as during suspension from the notum.

The definiteness of postural responses as indicated by the antennae may suggest that the antennae bear sense organs which are particularly susceptible to gravitational and centrifugal stimulation and thus are originators for postural reflexes. Thus it might be assumed that with the removal of the antennae the typical responses of an insect walking freely on a turntable might cease, or the postures of the abdomen might not be exhibited, when the animal is held fixed. No evidence to this effect could, however, be found. The typical responses persist after the antennae have been removed. There is no indication that the velocity of response or its extent is affected. At the same time the responses of the antennae are not affected when the abdomen is cut off. From recent work on proprioceptors in insects (Barnes, 1931; Pringle, 1938*a, b, c*), it is evident, however, that receptors responsible for postural reflexes are numerous and widely distributed over all parts of the insect's body, hence the mere removal of the antennae need not obliterate postural responses. On the other hand, there is strong evidence that the precision of orientation of the bee in its environmental field does involve functions of the antennae (Bethe, 1898, 1902; Wolf, 1926, 1927, 1930, 1931; Kalmus, 1937). Hence it can perhaps still be assumed that in the bee with antennae intact these organs play a part in the general exhibition of postural reflexes.

VI

In extending the observations on the bee, we also studied a series of other insects in respect to their antennal responses under gravitational and centrifugal force. In general, as far as a technical duplication of the tests was possible, the same reactions were found in several species of ants (*Formica, Lasius, Camponotus*) and in wasps (*Vespa maculata*). Among Coleoptera *Palidonota punctata* (*Scarabaeidae*) and *Pseudolucanus capriolus* (*Lucanidae*) gave clear antennal responses to rotation

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and to sidewise translation. In other *Scarabaeidae,* such as *Macrodactylus subspinosus,* no reactions were noticeable. *Chlaenious sericeus* (*Carabidae*) did not show any response. Among butterflies we have so far tested only *Pieris rapae* (*Papilionidae*); no response could be noticed. *Blattidae,* particularly *Cryptocercus punctulatus* (Sudder), show very nice responses if the animal is prevented from keeping its antennae in contact with any solid object. The question might be raised whether the appearance of definite postural responses of the antennae is in some way related to the insect's ordinary habits in general, or to its velocity of progression in flight or in walking. Such questions, however, could only be answered after a systematic study of many insects.

Summary

Bees respond to gravitational and centrifugal stimulation by characteristic movements and postures of their antennae. Depending upon the direction of motion in relation to the bee's body, one or both antennae point against the direction of movement. Together with the antennal postures, definite postures of the abdomen can be observed. Free running bees also exhibit postural responses of the antennae which are correlated with the direction of progression under the influence of centrifugal force. Removal of the antennae does not eliminate postural responses of the body. In several other insects similar postural responses can be observed, while in certain forms they do not appear.

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GROWTH STUDIES ON CILIATES

IV. The Influence of Food on the Structure and Growth of Glaucoma vorax sp. nov.

G. W. KIDDER, D. M. LILLY AND C. L. CLAFF

(*From the Arnold Biological Laboratory, Brown University, Providence, Rhode Island*)

Morphological variations which may be correlated with physiological condition have been noted in many different protozoa. It is well recognized that well fed protozoa are usually proportionately large while starved specimens may be quite small. Monsters of various types may result from certain cultural conditions such as certain bacterial diets (Kidder and Stuart, 1939) or from less well known influences during population decline. Among the hypotrichous ciliates one sees a marked change in size taking place when these forms become cannibalistic (Geise and Alden, 1938), but this increase in size is seldom accompanied by drastic morphological variations. The limits of morphological variation under varied conditions of food and other environmental factors must be recognized in order that the taxonomic validity of any species may be established.

The following report is based on the study of a hitherto undescribed species of *Glaucoma* (herein designated as *G. vorax sp. nov.*) and the experiments carried out were designed to gain some understanding of the food requirements, the mechanism of feeding and the factors influencing the profound structural changes of this interesting ciliate.

Material and Methods

The Ciliate

Glaucoma vorax was discovered in samples brought in from a small fresh water pond located in the vicinity of Providence, Rhode Island. In these samples large numbers of *Colpidium campylum* were also present. After remaining in the laboratory for a few days it was noted that the *Colpidium* were decreasing in number and a very large (150–250 μ) ciliate was becoming quite numerous. Upon isolation this ciliate, after two to three divisions, reverted to a slim, tailed *Glaucoma*, very similar in general appearance to *G. frontata*. These tailed forms were then placed in a suspension of our pure line *Colpidium campylum* and, within 24 hours, large forms were again present. It was apparent that the tailed *Glaucoma* had become carnivorous and were using the *Colpidium* for food. One of these large carnivores was selected for sterilization and it is from this organism that all of our stock lines have been derived.

Sterilization

The method of sterilization employed was a modification of that used by Parpart (1928) but the changes in manipulation appear to be worthy of description. Petri dishes were replaced by Syracuse watch glasses. Ten of these were placed individually in cellophane bags,[1] the open ends of the bags folded over several times and the whole sterilized in the autoclave. After these had cooled, 5 ml. of sterile distilled water was placed in one of the watch glasses, the bag being opened only enough to permit the entrance of the flamed lip of the tube containing the water. Following a few preliminary washes, the single large ciliate was placed in the watch glass by inserting the micropipette into the open end of the cellophane bag. The ciliate was allowed to swim about in the distilled water for ten minutes and was then transferred to a second similarly prepared dish.

The advantage of the cellophane bag as protection for the washing fluid is that the fluid or the watch glass is never exposed to the air from above, all manipulations being carried on from the side. Another important advantage is that the swimming organism can be watched under the dissecting microscope at all times because there is no condensation of water on the cellophane as there is on the cover of a Petri dish.

After the ciliate had been carried through five such transfers it was left in the fifth dish for twelve hours, a precaution against ingested, but viable spores. To this dish, however, had been added a loop of sterile *Colpidium campylum* taken from a proteose-peptone agar slant. This departure from the usual method is applicable only to carnivores, but it does obviate the difficulty of finishing with a starved ciliate. In this case the experimental ciliate fed and two divisions resulted, so that four large carnivores were present at the end of the twelve hours.

One of these four carnivores was carried through the remaining five dishes in the same manner as before. By the time the tenth dish was reached all of the *Colpidium* had been left behind. Yeast extract (1 per cent) was substituted for the distilled water in the tenth dish and the ciliate was left in this medium for forty-eight hours. Unlike *Colpidium*

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[1] The cellophane bags have been successfully used by one of us (D. M. L.) during the sterilization of a number of hypotrichous ciliates for nutritional studies. The results of these studies will appear shortly.

striatum (Elliott, 1933) and *Glaucoma ficaria* (Johnson, 1935), this ciliate did not appear to require any "acclimatization" to the broth but was able to utilize the dissolved proteins at once. After size reduction (which will be described later) it began to multiply, so that many ciliates were present in the dish after forty-eight hours.

A number of *Glaucoma* were transferred aseptically from the tenth dish into tubes of yeast extract and flourishing cultures resulted. From these tubes all of the regular checks for sterility were conducted (see Kidder and Stuart, 1939) with negative results. Agar plates have been poured from time to time, some incubated at 37° C. and some at room temperature, and all kept for a minimum of two weeks. There has been no indication of bacterial contamination in any of our stock lines.

Food Organisms

The bacteria-free ciliates and flagellates used as experimental food were obtained as follows: *Colpidium campylum* was sent to us by Dr. Robert H. Hall and is the strain investigated by him for oxygen consumption (Hall, 1938). *Colpidium striatum* was sent to us by Dr. A. M. Elliott and is the strain isolated by him in 1933. *Glaucoma pyriformis* was sent through the courtesy of Dr. Austin Phelps and is the strain investigated by him (Phelps, 1936). *Glaucoma ficaria* was isolated bacteria free in this laboratory, as was *Glaucoma scintillans* (the method employed in these isolations together with their growth characteristics will be reported later). *Astasia klebsii* in bacteria-free culture was obtained through the courtesy of Dr. Herman Von Dach. *Chilomonas paramecium* was supplied by Dr. J. B. Loefer and is the strain investigated by him in regard to the utilisation of dextrose (Loefer, 1938). *Euglena gracilis* was sterilized and established in culture in this laboratory. The yeast used was obtained by streaking from a Fleischmann yeast-cake onto proteose-peptone-dextrose agar plates and selecting isolated colonies for pure culture.

Techniques

For a study of the structure of *Glaucoma vorax* various preparations were made. The usual preparations (haematoxylin and the Feulgen reaction) for nuclei proved very satisfactory. For details of the cilia, ciliary lines, and mouth parts nigrosin and opal blue preparations gave beautiful results. A ciliate prepared by either of these relief methods is transparent to a degree so that the ciliary lines can be studied on both sides of the animal by focusing down from the top to the bottom surface. The silver line system was demonstrated in a satisfactory manner by using the dry method of Klein (1926).

Living material was observed and studied in hanging drop cultures. Under these conditions the ciliates remain healthy in appearance, feed, grow and multiply for many days. The hanging drops were prepared in the same manner as has been previously described (Kidder and Stuart, 1939*a*, 1939*b*) and were free from bacterial contamination.

OBSERVATIONS

Having at hand an unlimited supply of sterile saprozoic *Glaucoma vorax*, it was possible to test their ability to utilize certain common types of foods and to study the relative effects of these foods on structure and growth. The following account is largely qualitative, but observations on division rates have been made and will be indicated pending a more complete report to be given at a future date.

Bacteria

Microscopic examination of the ciliates from the wild had demonstrated the fact that bacteria were taken into the food vacuoles. We have made no attempt to determine what degree of species selectivity is practiced in nature, but have rather tested the influence of a single strain of living bacteria (*Aerobacter cloacae*) on the structure and growth of our experimental ciliate.

When washed, broth-grown ciliates are placed in a suspension of *Aerobacter* in distilled water they began to feed at once, small vacuoles form and the organisms resemble in every detail the thin tailed organisms found in nature (Fig. 1, *A*). These ciliates range from 50 μ to 75 μ in length and from 10 μ to 18 μ in width. Well fed ciliates appear quite granular, but as the bacteria are eaten out of the culture they become clear and refractive. Division of the tailed forms occurs and the daughter ciliates are also tailed. During the early periods of growth when adequate food organisms are present the interdivisional time is about six hours at 25° C.

Living bacteria as food have been considered first because this type of food is the one utilized by the ciliates in nature, at least to a large extent.

FIG. 1. Illustrations of *Glaucoma vorax* from life. × 500. *A*. Tailed form, bacteria-feeder. *B*. Broth-grown saprozoic form, during early period of growth. *C*. Small saprozoic form during the decline period of a broth culture. *D*. *Glaucoma* which has fed on dead *Colpidium*. *E*. Starved form from a dead *Colpidium* culture. *F–I*. Progressive form changes of saprozoic form (*B*) in the presence of living *Colpidium*. Note the formation of the preparatory vacuole and the size increase of the cell. *J*. A young carnivore removed to living yeast.

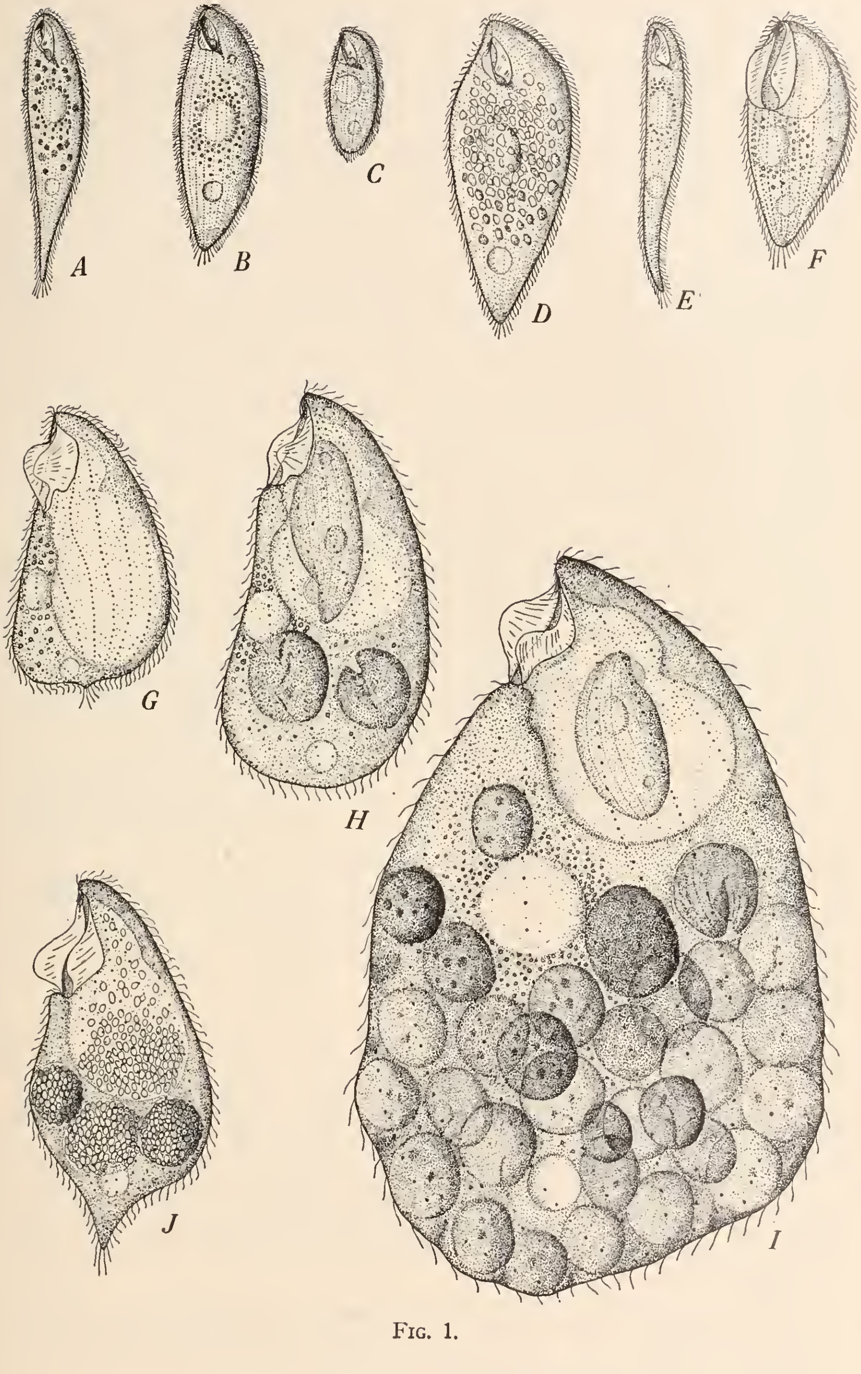
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Fig. 1.

Dissolved Proteins

Glaucoma vorax is able to utilize dissolved proteins as food and thereby to reproduce to relatively high concentrations per unit volume. We have grown this ciliate on the following media: proteose-peptone broth (0.5, 1.0, 2.0, 4.0 per cents); peptone broth (0.5, 1.0, 2.0 per cents); silk peptone broth (0.5, 1.0 per cents); tryptone broth (0.5, 1.0 per cents); yeast extract (0.5, 1.0 per cents by weight of Difco dehydrated yeast extract); yeast autolysate (1.0, 2.0, 5.0, 10.0 per cents by volume from a preparation made according to the directions given by Hetherington, 1932 and 1933 and used by Phelps, 1936). The addition of dextrose to some of the above media was made, but was found to inhibit growth to some degree. This is in contrast to the statement of Loefer (1938) that growth was better when dextrose was added to his basic medium and tested on *Colpidium, Glaucoma, Chilomonas* and *Chlorogonium.* The reason for this discrepancy is being more thoroughly investigated.

Sterile *Glaucoma* growing in any one of the above media become relatively clear and lose their distinctive tailed appearance (Fig. 1, *B;* Fig. 2, *A*). They range from 50 μ to 70 μ in length and from 15 μ to 22 μ in width, during the early stages of growth. After a period of days they gradually become reduced in size until the majority are only about 30 μ in length (Fig. 1, *C*). These saprozoic ciliates resemble to a marked degree *Colpidium campylum* growing in the same type of medium. The interdivisional period is approximately eight hours at 25° C. in most of the above media. This is, of course, very much longer than that of *Colpidium.* More detailed studies of the growth characteristics are being conducted and will be reported later.

When broth-grown ciliates are placed on proteose-peptone agar they form colonies and multiply to very high concentrations. This is a convenient way to carry stock cultures as a single agar slant may be kept for months and viable ciliates recovered.

Particulate Proteins

Autoclaved *Aerobacter* in distilled water is not adequate to support growth in *Glaucoma vorax.* The ciliates remain alive for long periods of time, but do not increase in size nor do they multiply.

Suspensions of autoclaved yeast support slow growth, but it is probable that the ciliates are taking in a certain proportion of dissolved proteins from the yeast cells as there does not appear to be active eating of the dead yeast cells.

Yeast Harris[2] (dehydrated) in 1 per cent by weight concentrations supports fairly good growth. The interdivisional period is approximately ten hours at 25° C., but the cultures remain in good condition for weeks. The maximum concentration in tubes of Yeast Harris eventually equals that of the proteose-peptone of yeast extract. The form of the ciliate in Yeast Harris is the same as the bacteria-feeder, tailed, and small food vacuoles containing the particles are scattered throughout the cytoplasm.

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[2] Brewers' Yeast—Harris. Pasteurized, dehydrated yeast cells prepared by the Harris Laboratories, Tuckahoe, New York.

Autoclaved *Colpidium campylum* (loops of colpidia taken from an agar slant, suspended in distilled water and autoclaved) and *C. campylum* shaken with sterile sand and not autoclaved serve as excellent food for the *Glaucoma*. The form changes are quite characteristic under these conditions and are identical for both autoclaved and fresh (sand-shaken) colpidia. The *Glaucoma* became proportionately much broader (30 μ to 40 μ). The pointed posterior end is evident though not so tail-like as in the bacteria-feeders and the cytoplasm becomes densely granular and quite dark (Fig. 1, *D*). Microscopic examination shows that the pieces of colpidia are taken into food vacuoles and become distributed throughout the cell, giving it its dark appearance. Interdivisional time is approximately 5.5 hours during the early period of growth when an adequate number of colpidia particles are present. Division of these darkly granular forms results in rounded daughter ciliates which rapidly assume the shape and size of the parent cell. After the particles of colpidia are depleted from the medium form changes take place in which the ciliates lose their granular appearance and become extremely thin (Fig. 1, *E*). These thin ciliates will persist in a tube for weeks without losing their activity. The concentration of living organisms gradually falls, but even after three months many viable forms may be recovered from the tube. It is probable that these remaining ciliates have been subsisting on the dead bodies of their sister cells.

Living Protozoa

If a sterile suspension of *Colpidium campylum* is prepared by placing a loopful from an agar slant in sterile distilled water or balanced salt solution and a few *Glaucoma vorax* are inoculated into this tube, within twenty-four hours large carnivores have made their appearance. The interesting form changes which occur can be followed best in hanging-drop preparations. In these latter preparations a single small *Glaucoma* placed in a heavy suspension of living colpidia may be followed by frequent observations under the medium powers of the compound microscope.

Within a few hours after the preparation is made the *Glaucoma* begins to broaden and the mouth becomes larger and more open. Back of the mouth a large clear space appears which is continuous with the outside through the mouth opening (Fig. 1, *F*). This is the start of the formation of the "preparatory vacuole" and probably represents the small vacuole of the tailed form which becomes enlarged and fused with the gullet. The "preparatory vacuole" increases in size until it nearly reaches the posterior end of the ciliate. At this period the vacuole occupies the greatest volume of the cell with the *Glaucoma* protoplasm surrounding it in a thin film (Fig. 1, *G*). Only after the completion of the formation of the "preparatory vacuole" can the *Glaucoma* feed on the live *Colpidium*. The first meal appears to be the most difficult to accomplish. As the mouth is open and large the membranes create strong currents into the "preparatory vacuole," eventually a *Colpidium* is drawn in. The *Glaucoma* immediately becomes quite active and swims in circles with the mouth directed toward the inner part of the circle. This first prey swims about in the vacuole in an entirely normal manner and may eventually swim out of the mouth and escape. Sometimes two or more colpidia are drawn in together (Fig. 2, *E*). Eventually the *Glaucoma* protoplasm closes down until the "preparatory vacuole," with its trapped *Colpidium,* is cut off from the mouth region. The enclosed prey continues to swim about, but the fluid content of the vacuole decreases slowly and the protoplasms of the carnivore and prey come to lie close together and the motion of the latter is restricted. After about twenty minutes the prey has lost all activity and digestion is under way. As digestion proceeds the *Glaucoma* increases in size and from that time on it is able to capture colpidia much more rapidly (Fig. 1, *H,* 2, *B–E*). Within the space of an hour 40 to 50 colpidia may be captured and the resulting growth of the *Glaucoma* may bring its length up to 250 μ and its width to as much as 150 μ (Fig. 1, *I*). These large carnivores eventually divide transversely (Fig. 2, *F*), each daughter ciliate carrying over about half of the food inclusions. After separation the daughter ciliates redifferentiate, feed, grow in size and again divide. This process is repeated until the colpidia in the medium become scarce. The *Glaucoma* continue to divide, but each daughter cell is smaller until the typical tailed form is again attained. As long as there are any colpidia present, however, the "preparatory vacuole" is retained in at

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FIG. 2. Photomicrographs of living *Glaucoma vorax*. These pictures were (with the exception of *A*) made from a hanging drop preparation in which, forty-eight hours previous to photographing, one small saprozoic *Glaucoma* was transferred aseptically to a suspension of living *Colpidium*. *A*. Saprozoic ciliate. × 320. *B*. Carnivore showing enlarged mouth and rounded food inclusions (*Colpidium*). × 180. *C*. Same, but with many more food inclusions. × 180. *D*. One carnivore with a few inclusions to show the relative size of the *Glaucoma* and the prey. × 60. *E*. Carnivore showing the preparatory vacuole in which two colpidia are trapped. × 250. *F*. Division of a carnivore. × 180.

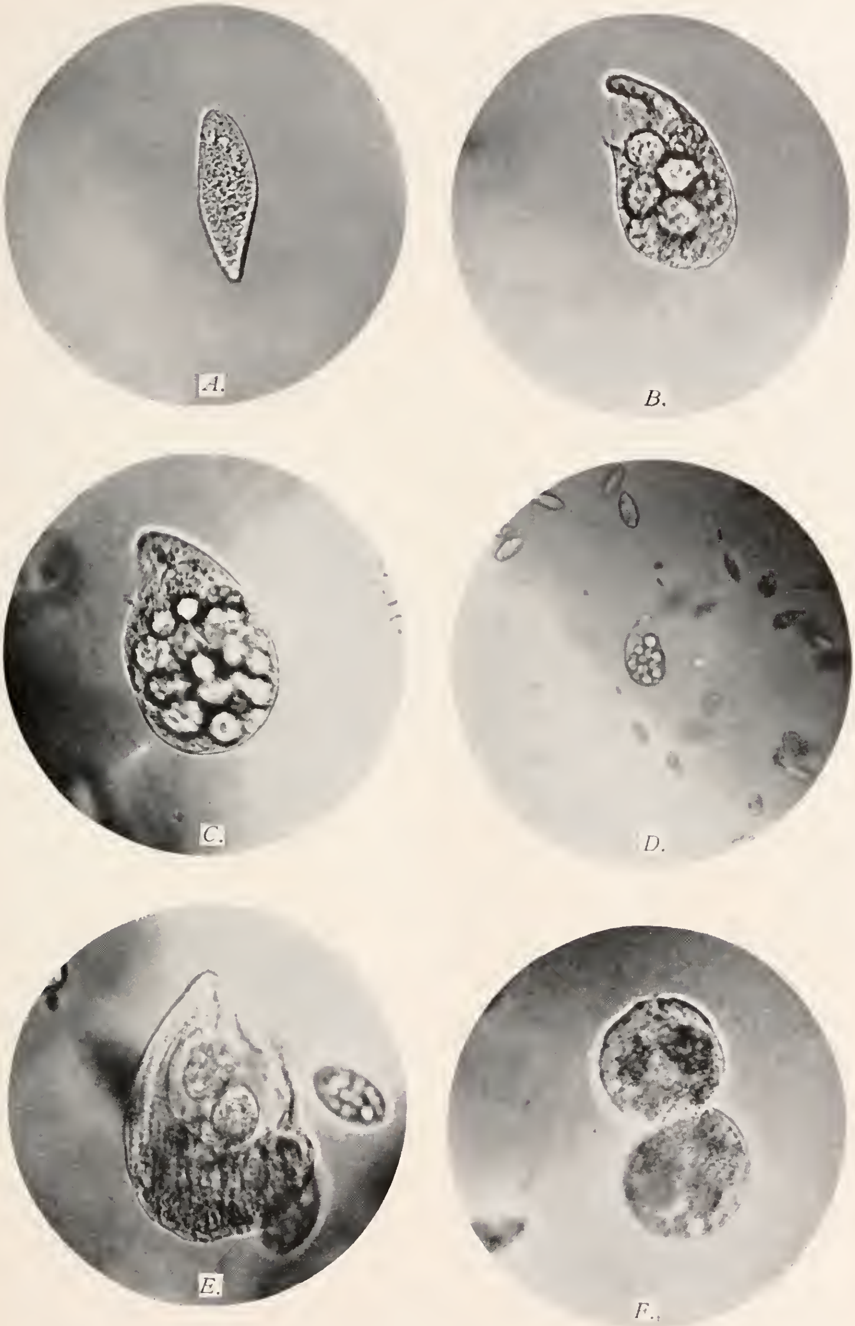

Fig. 2.

least some of the *Glaucoma* so that eventually all of the colpidia are eaten. We have recorded many cases in hanging drops where the ratio of *Colpidium* to *Glaucoma* at the start was about 2000:1 and after four to five days many *Glaucoma* were present and no *Colpidium*. Likewise the *Glaucoma* are able to clear out the colpidia from a tube until none can be found after five to six days.

These large carnivores are the type which we encountered in our original pond sample, one of which was used for sterilization.

The formation of the preparatory vacuole is not dependent upon anything peculiar to *Colpidium campylum*, however, as exactly the same response is evoked by the presence of all of the ciliates tried. These were *Colpidium striatum* (Elliott strain), *Glaucoma pyriformis* (Phelps strain), *Glaucoma ficaria* (our strain), *Glaucoma scintillans* (our strain) and *Colpoda* (our (Kidder and Stuart, 1939) strain grown on *Aerobacter*). Cannibalism also occurs, especially in broth cultures. After the concentration of a pure culture of *Glaucoma vorax* has reached a value somewhere between 15,000 and 25,000 ciliates per ml. a few organisms assume the form described for the *Colpidium*-feeders and begin eating members of their own species. The proportion of cannibals to non-cannibals is always very low, however, for after one or two divisions these forms revert to the typical saprozoic types.

Living Yeast

If saprozoic ciliates are placed in a suspension of living yeast they appear to be unable to ingest the yeast cells. They live for a week or more but finally disappear from the culture. No form change ensues except for a decrease in size due to starvation. If, however, a carnivore is placed in a like suspension it is able to eat the yeast cells. The yeast is concentrated in the preparatory vacuole in large balls containing hundreds of cells and then the vacuole is separated from the mouth region in the same manner as previously described for colpidia-feeders. These yeast-filled *Glaucoma* (Fig. 1, *J*) grow slowly and divide, the daughter ciliates again feeding as had the parent. The yeast-feeders never reach the large size of the carnivores and reversion to a tailed condition usually takes place long before the food is exhausted.

Living Flagellates

We have tested the effect of the following bacteria-free flagellates on *Glaucoma vorax: Astasia klebsii, Chilomonas paramecium* and *Euglena gracilis*. Saprozoic *Glaucoma* placed in a washed suspension of any one of the above flagellates slowly starve and disappear from the culture. As with the yeast cells, there is no alteration of form. However, pre-formed carnivores feed on the flagellates. Both the feeding mechanism and the reproduction of the *Glaucoma* are slowed down under these conditions. The flagellates are ingested sparingly and the ciliates do not increase appreciably in size, even after many hours. One, or sometimes two, divisions occur and reversion to the tailed condition ensues. It is possible that this type of food is entirely insufficient for reproduction and the one or two divisions noted result from a carry over of nutrient materials within the ciliates from their previous environment.

Discussion

Glaucoma vorax exhibits the most varied food-taking habits of any known species of the genus. Like a number of other species within the genus it may be a bacteria-feeder or, when deprived of its associated bacteria, utilize dissolved proteins. On the other hand, it resembles to a marked degree *Leucophrys patula* in that it can ingest and digest relatively large ciliates. In order to do this, however, drastic form changes must take place. In *Leucophrys* the large receiving vacuole appears to be a permanent structure (M. G. Brown, unpublished observation) and when the food ciliates are exhausted from the medium there is no change in form but simply a diminution of size. *Leucophrys* appears to be more strictly an obligate carnivore.

One of the most significant facts about *Glaucoma vorax* is the formation of the preparatory vacuole previous to the ingestion of living protozoa. This formation appears to be stimulated by the presence within the medium of living ciliates. We have made a number of preliminary experiments in an attempt to determine what factor or combination of factors may be operating. Extracts from *Colpidium* were placed in the medium. These extracts were prepared by cutting up the colpidia by shaking with sand and passing the fluid through a Seitz filter. The filtrate was entirely without effect on the form of the saprozoic *Glaucoma*. The dead bodies of *Colpidium*, although eaten by the *Glaucoma*, fail to evoke the vacuole formation. Pieces of fresh *Colpidium*, shaken with sterile sand, were eaten but did not cause vacuole formation. Live flagellates and yeast failed to evoke the vacuole formation although a pre-formed carnivore was capable of ingesting these organisms. Although investigations which are now being carried on may clear up our understanding of the factors involved in this important cell mechanism, we can only say at the present that the stimulus necessary for the preparatory vacuole formation and the accompanying form changes appears to reside in the living, actively moving ciliate. It may be that the stimulus is of a mechanical nature and that any cell possessing the right size and degree of activity will evoke the response.

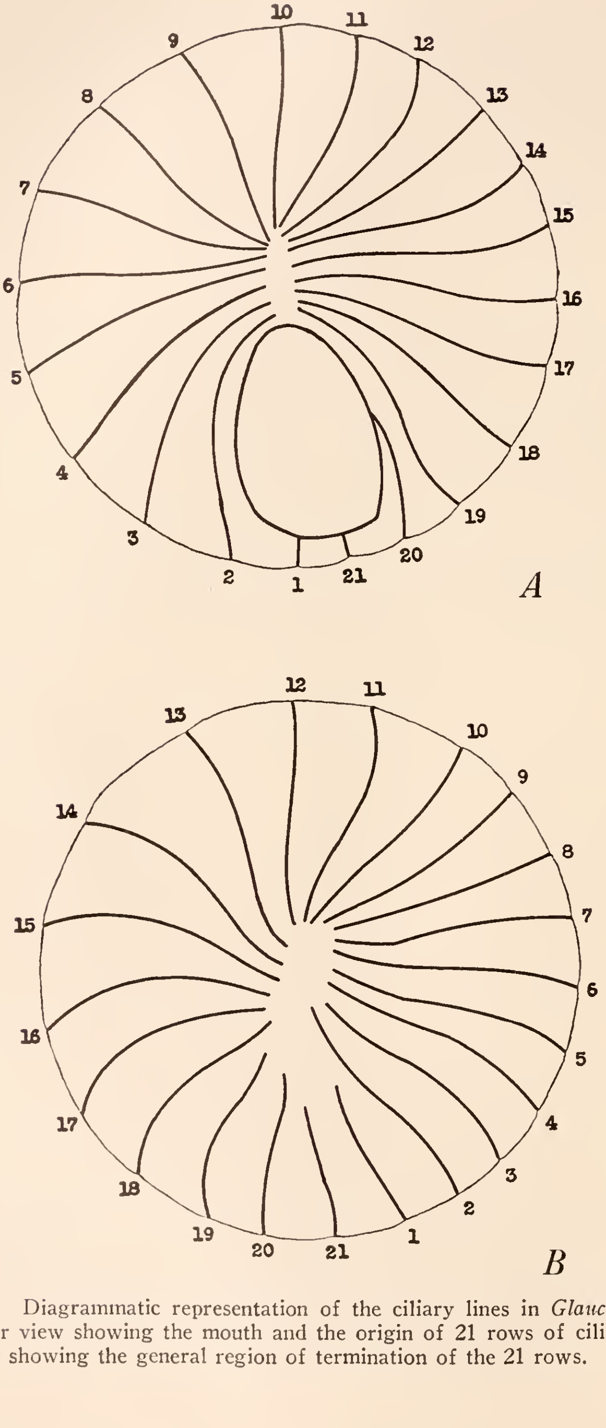
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Fig. 3. Diagrammatic representation of the ciliary lines in *Glaucoma vorax*. *A*. Anterior view showing the mouth and the origin of 21 rows of cilia. *B*. Posterior view showing the general region of termination of the 21 rows.

Glaucoma vorax seems to offer exceptional opportunities for investigations into the mechanisms of feeding, specific food requirements including accessory factors, the growth characteristics under varied conditions and the further effects of associated organisms on structure and metabolism.

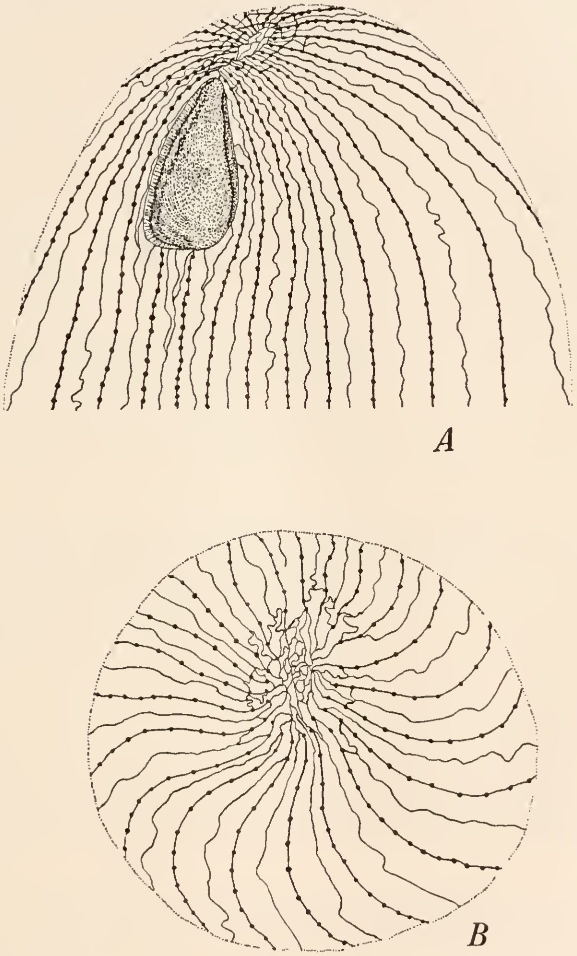

Fig. 4. Silverline system of *Glaucoma vorax*. *A*. Anterior end showing mouth and anterior suture. Note interstrial fibers. *B*. Posterior end showing network of fibers in the posterior suture.

Species Diagnosis of Glaucoma vorax sp. nov.

1. Sizes: Bacteria-feeders 50 μ to 75 μ; saprozoic forms 30 μ to 70 μ, the size decreasing with the age of the culture; sterile particle-feeders

(autoclaved or otherwise killed ciliates) 60 μ to 80 μ; carnivores and cannibals 100 μ to 250 μ, depending upon the amount of food taken.

2. Shape: Varies enormously depending upon the amount and type of food taken. Bacteria-feeders elongate with a distinct tail process; saprozoic forms spindle-shaped to ovoid; carnivores and cannibals distended posteriorly and usually irregular in outline due to the large food inclusions (see illustrations).

3. Cytostome: Typical for the genus except in the case of the carnivores and cannibals where it is vastly distended and continuous with a large, internal preparatory vacuole for the reception of prey. Cytostomal membranes typical for the genus.

4. Body cilia: Disposed in from 19 to 21 rows. Sixteen to 18 of these rows originate in an anterior suture (Fig. 3, *A*) while three originate from the border of the cytostome. These last three are the shortest rows as they terminate short of the posterior field (Fig. 3, *B*). All of the others terminate in the posterior field. Each row bears a series of basal bodies from each of which arises a single cilium. In the carnivores the spacing of the rows is in the same relation as in the tailed forms, but the distance between rows is much greater (Fig. 1, *F–I*).

5. Contractile vacuole: Single and located in the posterior third of the body.

6. Nuclear apparatus: A single ovoidal macronucleus located in the mid-region of the body. The macronucleus increases in size with the growth of the ciliate (carnivore) but becomes slightly irregular in outline and progressively less basophilic. A single micronucleus, located near the macronucleus.

7. Reproduction: Binary fission in all form types.

8. Conjugation: Never observed in our strain.

9. Silverline system: Characteristic pattern (Fig. 4, *A* and *B*) with network of fibers in the anterior and posterior sutures and with irregular interstrial fibers between the longitudinal fibers which connect the basal bodies.

10. Food: In nature bacteria and ciliates, possibly members of the same species (cannibalistic). Our strain is able to feed on a variety of particulate and non-particulate proteins. Carnivores produced in the presence of living ciliates of any one of a number of different species. Live yeast and certain flagellates may serve poorly as food provided they can be ingested.

11. Type strain: May be procured bacteria-free from our laboratory.

Summary

1. *Glaucoma vorax* sp. nov. was isolated from pond water in the vicinity of Providence, R. I.

2. It was sterilized and established in broth culture by a modification of the transfer washing method in which the containers were enclosed in cellophane bags before autoclaving.

3. Various form changes were correlated with types of food; bacteria-feeders are tailed, saprozoic organisms are spindle-shaped to ovoidal while carnivores and cannibals are irregularly ovoid with the greatest width at the posterior end.

4. When saprozoic forms are placed in a culture or washed suspension of other ciliates (*Colpidium, Glaucoma, Colpoda*) they change their form preparatory to becoming carnivorous. A large vacuole forms back of and continuous with the mouth to receive the prey.

5. The formation of the preparatory vacuole and the concomitant form changes seem to be evoked only in the presence of living ciliates, as killed ciliates (either autoclaved or freshly sand-shaken), living flagellates, bacteria or yeasts do not stimulate its formation.

6. A diagnosis of the species is given.

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HISTOLOGY OF THE RETRACTOR MUSCLES OF PHASCOLOSOMA GOULDII POURTALÈS

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Although much work has been done on the detailed anatomy of the invertebrates, very little more than incidental and superficial descriptions of the musculature have been given. Inasmuch as many invertebrate muscles are being used in physiological and biophysical research, it seems important that a knowledge of their detailed histology be made available. As I indicated in a previous paper (Olson, 1938), histological studies of the muscles of the invertebrate series may also reveal facts of possible phylogenetic significance.

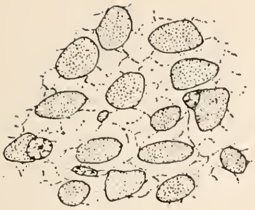
I have found in the literature no previous detailed histological studies of the retractor muscles of *Phascolosoma* although Gerould (1906) mentions the formation of muscle fibers in his description of the development of *Phascolosoma.* Descriptions of muscles of related forms (Graff (1875), on *Chaetoderma;* Andreae (1881), on *Sipunculus nudus;* Apel (1885), on *Priapulus* and *Holicryptus;* and Jameson (1899), on *Thalassema*) present quite a different histological picture.

Materials and Methods

The retractor muscles of *Phascolosoma* consist of two pairs of flattened bands which extend from the front end of the base of the introvert to the body wall. They are 3–5 mm. in width and 1–2 mm. in thickness. They vary in length from 1–1.5 cm. in normal contraction to 7–10 cm. in normal extension. The ventral retractors are somewhat longer than the dorsal, being attached 3–5 mm. posterior to them. They are also somewhat thicker.

The muscles were fixed in varying states of contraction in Bouin's and Helly's fixing fluids. They were dehydrated in Dioxan, imbedded in paraffin, and sectioned at 5 and 10 micra. The sections were stained with Heidenhain's iron haematoxylin, Mallory's phosphotungstic acid haematoxylin, and Dominici's staining mixture.

The muscles were successfully macerated in 10 per cent nitric acid. It was thus possible to obtain free fibers and to measure fiber lengths.



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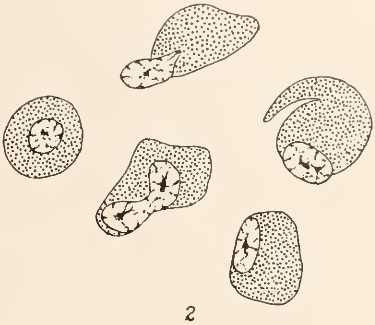

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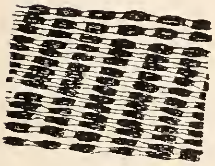

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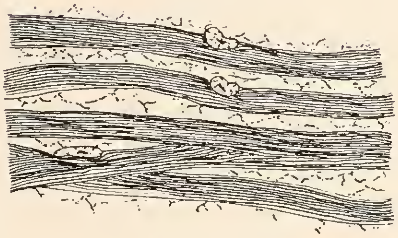

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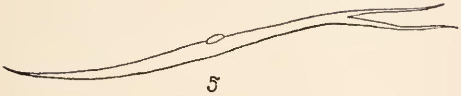

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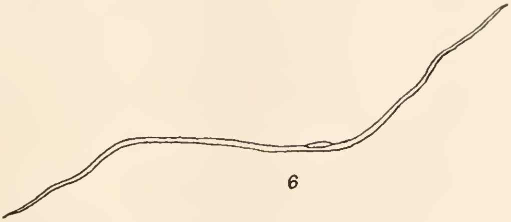

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EXPLANATION OF FIGURES

All drawings were made with the aid of a camera lucida.

FIG. 1. Cross-section of a small portion of a muscle showing fibers, fibrils, and distribution of connective tissue. × 625.

FIG. 2. Cross-section of individual fibers showing the position of the nuclei. × 1250.

FIG. 3. Longitudinal section of a portion of a muscle fixed in isometric contraction showing the cross-striations produced by the vertical alignment of the contraction nodes of adjacent fibers. × 168.

FIG. 4. Longitudinal section of a portion of a resting muscle showing fibers, fibrils, fiber nuclei, and the distribution of the connective tissue. × 625.

FIG. 5. A contracted branched fiber from a preparation of macerated muscle. × 63.

FIG. 6. A typical resting fiber from a preparation of macerated muscle. × 63.

Results

The retractor muscles of *Phascolosoma* consist of comparatively densely packed spindle-shaped fibers which in the resting state have an average length of about 1 mm. and a maximum length of about 1.5 mm. The fibers vary in diameter from 3 or 4 micra up to 20 micra with an average diameter of about 6 or 7 micra. In cross-section the fibers may appear cylindrical, hemicylindrical, or angular. Occasional branched fibers are also found (Cross-section, Fig. 2; single fiber from teased muscle, Fig. 5).

The fibers are uninucleate, the nuclei occupying a position similar to the nuclei of vertebrate smooth muscle midway between the terminals of the fibers (Fig. 6). Unlike the nuclei of vertebrate smooth muscle, however, they usually have an eccentric position within the fiber (Fig. 2). Occasional nuclei are found in a central position, a few others peripheral to the fibrillar zone.

The contractile elements are densely packed fibrils uniformly distributed throughout the fiber, and not peripherally arranged as in *Thalassema* (Jameson, 1899), *Sipunculus* (Andreae, 1881), *Priapulus* and *Holicryptus* (Apel, 1885). They are essentially straight parallel structures as described by Keferstein (1865), and in no instance show the spiral structure described by Jameson for *Thalassema*. The fibers fail to show the multiplicity of zones described for the muscle cells of other sipunculids. A central canal, an area of fibril-free sarcoplasm, can not be demonstrated. The description by Jameson of radially arranged contractile plates in the cortical layer and an outer hyaline layer bounding the sarcolemma externally could likewise not be substantiated in a study of the retractor muscles of *Phascolosoma*.

The muscle fibers do not show a definite arrangement into bundles as do the retractor muscles of *Thyone* (Olson, 1938). The proportion of connective tissue and tissue space is also considerably smaller than in *Thyone*. As in the retractor muscles of *Thyone,* however, each fiber is invested by connective tissue which forms a delicate reticulum connecting adjacent fibers (Figs. 1 and 4).

A curious phenomenon not previously reported for invertebrate muscle is the cross-banded appearance of the muscle fixed in a state of isometric contraction (Fig. 3). The " striations " are visible only in muscles fixed in isometric contraction; the resting and isotonically contracted muscles do not show them. The cross-banded muscles resemble the so-called striated muscles which Carey (1921, 1924) claims to have produced experimentally in the bladder of dogs. Jordan (1938), however, believes the simulacra of cross-striations seen by Carey to have been produced by the alignment of contraction nodes, or in some instances, by the folding and buckling of the fibers. In the retractor muscles of *Phascolosoma* likewise, the dark and light bands can scarcely be homologized with the Q and J discs of striated muscle since they exceed in relative size the discs of any type of striated muscle, and further, do not show the characteristic Z membranes or M discs. They are best interpreted as being formed by the alignment of the contraction nodes of adjacent fibers, and inherently by the vertical alignment of the darkly stained areas of the fibrils within each muscle cell. However, the very fact of the precise alignment and the uniformity of the nodes suggests a primitive or simplified type of striated muscle or a transition type in a possible evolution of striated from smooth muscle. Hursh (1938) found that *Phascolosoma* retractor muscles, much as striated muscles, respond well to electrical stimulation and are able to shorten from one-sixth to one-seventh of their original length. The final interpretation, however, must await additional physiological data for corroboration.

Summary

The retractor muscles of *Phascolosoma* consist of densely packed spindle-shaped fibers which have an average length of 1 mm. and an average diameter of 6 or 7 micra.

The fibers are uninucleate, the nuclei usually occupying an eccentric position midway between the fiber terminals.

Fine fibrils are densely and uniformly distributed throughout the fibers. They are essentially straight parallel structures.

Vertical alignment of the contraction nodes of adjacent fibers in isometrically contracted muscle produces cross-striations simulating the Q and J discs of skeletal muscle.

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EFFECT OF COLCHICINE ON THE DEVELOPMENT OF THE FISH EMBRYO, ORYZIAS LATIPES

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I

The alkaloid, colchicine, has recently come into prominence biologically because of its effects on mitosis. In plants it produces polyploidy, while in animals it not only checks mitosis (Allen et al., 1937) but also appears to stimulate the mitotic rate (Paff, 1939; Havas, 1939; Mills, 1939). When introduced into the hen's egg of 48-hour incubation (Paff), the resultant effects include distortion due to overgrowth and abnormalities of the vascular system and body flexures. In the frog embryo, colchicine provokes a meroblastic type of cleavage, disturbs or limits gastrulation and produces enlarged cells (Keppel and Dawson, 1939). The embryos did not develop beyond the neural fold stage. Bastenie and Zylberszac (1938) have shown that it stimulates the parathyroid gland, while in male fish, Havas (1939) has found that colchicine provokes color change of the same intensity as testis hormone.

This paper is a report of colchicine effects upon the development of the fish embryo, *Oryzias latipes*. Attention was focussed particularly upon the differentiation of embryonic structures. All cultures were followed until the embryos either hatched or died. Exposure in the dark at room temperature was begun both before and after the onset of differentiation to different concentrations and for varying periods of time, after which the embryos were transferred to fresh water. In some cases exposure was continuous, while in others the chorion was pricked. During a period of continuous exposure in the dark, the colchicine solution was changed every 2–3 days.

II

Table I includes an outline of the experiments with cleavage stages together with a brief summary of the results based upon the use of toxic concentrations of the alkaloid. Non-toxic concentrations showed no stimulatory action on the development of this fish. In a 1–100,000 concentration development from a cleavage stage was only slightly retarded

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[1] This study was aided by a grant from the Rockefeller Foundation.

during a continuous exposure and the embryos hatched about 3 to 5 days later than the control. Increasingly stronger concentrations caused progressively greater retardation and the weakened embryos failed to hatch.

TABLE I

Effect of colchicine on early development of Oryzias latipes.
(Cleavage stages)

Developmental stage	Conc.	Length of exposure	No. eggs used	No. living at end of exposure	No. living at end of experiment	Time in fresh water	Results
Early cleavage	1–100,000	25 days	23		18		7 hatched by 25 days, weak and died within a few hours.
	1–10,000	10 days	14	none		3 days	Young optic cup stage. Trunk region reduced and tail absent. Dead.
	1–5,000	9–48 hrs.	82	probably 34		16 hrs.	Little differentiation. Increase in cell number and some axial thickening. Few in neural tube or optic vesicle stages. Died.
Medium cleavage	1–100,000	12–25 days	47	30			Slight retardation only. 7 hatched by 25 days.
	1–10,000	13 days	52	7		10 days	Small size. Reduced or spindly trunk and tail. Somites obscure. Trunk queerly bent. Yolk vascularization reduced or absent. One had weakly beating heart.
	1–5,000	2 hrs.	26	all	24	25 days	No retardation evident. 7 hatched by 22 days
		4 hrs.	35		35	30 days	Slight retardation. 8 hatched by 27 days.
Medium cleavage	1–5,000	20 hrs.				55 hrs.	All dead. Some had developed to optic vesicle stage with reduced tails.
		27 hrs.	93	43	none	19–33 hrs.	Many failed to recover. Few developed to non-pigmented eye stage. Trunk of varying length. All dead by 10 days.
		44 hrs.				31 hrs.	All dead. No recognizable differentiation.
Late cleavage	1–5,000	22.5 hrs.	67	60	35	21 hrs.	Only few more cleavages during exposure. After transfer = 7 in embryonic shield stage, 6 undifferentiated, 4 in short neural tube stage. Living remainder only slightly retarded.
		7 days	34	none			Optic vesicle to young optic cup stage. Reduced trunk and no tail development. Died.
	1–1,000	1 hr.	15	all?	4	8 days	Typical appearance in 4. Others died.
		2 hrs.	21	all?	6	8 days	3 retarded but typical. 4 with reduced trunk and tail, cyclopian-like eye, abnormal brain.
		3.5 hrs.	29	all?	none	2 days	No development in majority. 6 are in short neural tube stage.
			18	all?	6	7 days	5 retarded, 2 cyclopian cases, 5 lack tail with short trunk, small eyes with little pigment.
		9.5 hrs.	23	all?	none	7 days	No differentiation and no recovery.
		19 hrs.	52	all?	1	1 day	Further cleavage but no differentiation. Those left in colchicine are dead.

One to 10,000 caused great general retardation of size, differentiation, developmental rate, pigmentation of the eye and body, etc. It also inhibited and retarded the growth of the trunk region and tail, the formation of somites and extra-embryonic vascular vessels. The latter effect has been reported for the chick embryo by Paff (*loc. cit.*) who began exposure at the 48-hour stage. In many fish the rudimentary tail was strangely bent or twisted. Apparently there occurred a greater interference with the development of the trunk and especially the tail than with the head region. Following an exposure to 1–5,000 these effects were much accentuated while the tail might be entirely lacking. This solution acted very quickly since during exposures of 23 to 48 hours only a few cleavages took place while the eggs were in solution. The yolk became more or less covered by a cellular layer but no differentiation occurred. Embryos may adapt themselves somewhat to continuous exposure at this concentration and stage, but, as will appear later, older embryos showed greater capacity in this respect and even in a 1–1,000 concentration differentiation continued.

Exposure of cleavage stages for as short a period as 2 hours to a concentration of 1–1,000 subsequently produced small embryos with reduced trunk and tail, cyclopian-like eyes, reduced and uneven distribution of pigment in eyes and body, and abnormal brain which was frequently bent or folded. During the exposure period further development was limited to a few cleavages. About 4 hours later the majority failed to recover, while in fresh water a few embryos went as far as a shortened neural tube stage. The effect on the eyes was especially striking. The pigmentation was much reduced or lacking, while in the cyclopian-like eyes the pigment appeared as small, densely aggregated, irregularly-shaped masses at the anterior region. These masses varied from a large single one to two variously sized smaller ones connected by a line of pigment. A few such cases were secured from late cleavage stages but not from stages in which differentiation had begun.

The production of cyclopian eyes in animals has been discussed by Adelmann (1936) and apparently a wide variety of chemical substances are causative agents. Stockard (see Adelmann) concluded from his experiments on *Fundulus* that the action of chemicals must be exerted relatively early since eggs treated later than 15 hours after fertilization failed to respond, the most effective period being the 8–32 cell stage. The present results agree with this conclusion as more cases were secured from the earliest cleavage stages. Certain of the types of developmental modification produced in the fish embryo are comparable to those caused by x-rays and 2,4-dinitrophenol (Waterman, 1939).

The effect of colchicine is related to the concentration, length of exposure, and the stage of development. Three other developmental stages have been tested for comparison with the cleavage stages, i.e. the neural fold or tube, the optic vesicle and the optic cup stage previous to the appearance of pigment. Reference to Table II will show that these older stages have progressively greater survival capacity for comparable concentrations and that growth and differentiation continue, but more slowly than normal, during exposure to the strongest solutions employed. Fewer embryos died and the percentage of recovery was greater than in the case of the cleavage stages. All stages tested exhibited considerable variation in susceptibility of the individuals in a culture but this became

TABLE II

Effect of colchicine on early development of Oryzias latipes.
(Neural fold to optic cup stages)

Developmental stage	Conc.	Length of exposure	No. eggs used	No. living at end of exposure	No. living at end of experiment	Time in fresh water	Results
Neural fold	1–5,000	23 hrs.	43	all		20 days	23 hatched. Little if any retardation.
	1–1,000	11 hrs.	57	all?	9	5 days	Reduced or abnormal trunk and tail, no extra embryonic vascular vessels. Tail often lacking.
		21 hrs.	48	all?	none	2 days	In solution = retarded, reduced tail and small trunk, optic cup stage lacking pigment. Died.
		23.5 hrs.	41	all?	4	5 days	Development in sol. In fresh water one was typical, but small. 3 had reduced tails. Died.
Neural tube	1–1,000	13 hrs.	52		21	76 hrs.	Cyclopian-like distribution of eye pigment. Large ear vesicles. Tail reduced or absent. Small amount of body pigment. Very abnormal.
Neural tube to optic vesicle	1–1,000	13 hrs.	72	all?	31	3 days	Very retarded, reduced trunk and tail regions, very abnormal. Cyclopian-like distribution of eye pigment. Degeneration and regional differentiation common.
Non-pigmented optic cup beating heart	1–1,000	11 hrs.	35	all?	33	23 days	Retarded but some typical. Circulation abnormal or absent in more retarded cases. 6 hatched.
		27 hrs.	41	all			Slight heart beat, further development. Slight pigmentation of eyes. Larger size. None hatched.
		33 hrs.	20	all?	19		Retarded but typical. Small. None hatched.
Optic cup	1–1,000	23 hrs.	19	all	all	25 days	Typical but small. Feeble heart beat. Yolk plexus either typical, absent or showing no circulation. Died—none hatched.
		21 hrs.	33	all		10 days	Some further development. No pigment in eyes or body. Died.
		39–48 hrs.	68	all			Some differentiation during exposure. Slight pigment formation. No yolk-sac circulation. Enlarged pericardial sac. Shortened tail. None hatched.

less marked in the oldest stages. As in the case of the cleavage stages, effects were apparent in the size of embryos, body shape, pigmentation of eyes and body, development of the tail and in the circulatory system, especially heart and yolk circulation.

A common occurrence following the exposure of younger stages to a 1–1,000 concentration was an apparent degeneration of the embryonic tissues to form a dark mass of structureless material on the surface of the yolk. At first these cases were discarded but later, on the chance of possible recovery, they were transferred to fresh water. In one case very little change was noted until 2 to 3 days following transfer when observation showed aggregated eye-pigment masses and a variable distribution of pigment cells throughout the mass. In some the heart tube had formed and was beating feebly. The slight pulsation waves originated in the posterior part of the tube and proceeded anteriorly. Large vesicle-like cavities were common. These differentiations point to specific regional survival independent of normal association. No further development occurred although the cases survived many days.

Disturbances in the vascular system often account for the early death of otherwise quite typical embryos (Paff, 1939). Generally this appeared as a failure of the yolk blood vessels to form or there were evident isolated aggregates of pink or colorless blood cells on the surface of the yolk. In more retarded cases the heart failed to form, beat very feebly without any blood passing through it, was curiously bent, twisted or looped, or departed at odd angles from the body edge. In such instances it was always contained within a large cyst-like space beneath the head.

The central nervous system, especially the brain, appeared to be less affected although irregularities appeared even here. The neural tube often was bent or folded particularly in the mid-brain region. Vesicular outgrowths of the brain were common and different portions developed disproportionately, especially the cerebral hemispheres and cerebellum. As has been mentioned above, the development of the tail was retarded or even inhibited when the remainder of the embryo appeared quite typical. In extreme cases the tail was lacking and a corresponding shortening of the trunk region followed, or the tail resembled a spindle-like rudiment often bent in peculiar ways.

As in the chick (Paff), body flexures may be obliterated or accentuated producing weird results. In the light of the information that colchicine affects mitosis, such malformations and retardations as have been described above may in part at least be the result of this effect together with irregularities in differentiation. However, these effects are not uniform throughout all parts of the developing embryo, as differential susceptibility is exhibited by different organs and regions.

SUMMARY

1. The action of colchicine on the differentiation of embryonic structures of the fish embryo has been studied by exposing four different developmental stages of *Oryzias latipes,* i.e. early cleavage to optic cup stages, to various concentrations for different lengths of time.

2. The sensitivity of both the whole organism and its constituent parts decreases with age and differentiation. Abnormalities were produced whose type and degree depend upon the concentration of the alkaloid, length of exposure and the stage of development. Concentrations of 1–100,000 to 1–1,000 were employed but the latter proved to be most useful.

3. In addition to general retardative and inhibitory effects on cleavage and development, other types of abnormalities included variation in the pigmentation of the eyes and body, cyclopian-like eyes, retardation and inhibition of the development of the tail, irregularities in the heart and yolk-sac vessels, abnormal blood formation, disturbance of body flexures especially the tail, and disturbances in the development of parts of the brain. Apparently when gastrulation occurred, it was typical.

4. In cases where development had ceased entirely during exposure and slight degeneration had set in, differential recovery was seen to take place especially in the formation of pigment and heart development.

5. The alkaloid penetrates the egg chorion very quickly. In weaker concentrations the older embryos showed some adaptation during long exposures. Some of the described effects are similar to those caused by dinitrophenol and x-rays.

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EXPERIMENTS ON LIGIA IN BERMUDA

VII. Further Effects of Sodium, Ammonium and Magnesium

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This paper is a continuation of a series dealing with the ecology and physiology of the littoral isopod *Ligia baudiniana.* In spite of its terrestrial life this interesting transitional form is morphologically equipped for life in the sea. The gills are well developed and their beating in sea water has temperature characteristics typical for respiratory processes (Barnes, 1936). The experiments were designed to determine how *Ligia* survives in air, what factors restrict it to the littoral zone and how long the animal survives in modifications of sea water. A brief summary of previous results will serve to indicate the significance of the new data presented below. Without entering the sea the *Ligia* draws water to the gills by capillarity (Barnes, 1938) but the young are released under water (Barnes, 1932). *Ligia* orients to the shore by negative phototropism in the sea and by positive geotropism on land (Barnes, 1932, 1935). Under experimental conditions *Ligia* survives about two days in natural sea water and in 25 per cent sea water with added calcium (Barnes, 1938). No artificial sea water more favorable than natural sea water has been found (Barnes, 1936). The toxicity series for the common cations in sea water is typical of other Crustacea (Barnes, 1932). The isopod shows a marked negative reaction to filter paper moistened with sea water (compared with distilled water). It was shown that this is probably an effect of sodium (Barnes, 1939). In brief, these studies aim to describe some of the factors regulating the life of a marine form which has recently invaded the intertidal zone.

Reactions to Filter Paper Moistened with Salt Solutions

To determine which ion in sea water repels *Ligia* from filter paper moistened with this medium, it was necessary to test the common ions of sea water separately. Previous experiments (Barnes, 1939) showed that Ca has no repellent action; and the concentration of K in sea water is below the threshold for the negative reaction. It remained to test Mg, the commonest ion after Na. As in previous experiments a filter paper 25 cm. in diameter was cut in two and each half saturated with a different medium. As will be seen in Table I, an equal distribution of isopods occurred between $MgCl_2$ and distilled water papers. Since this shows that magnesium, like calcium and potassium, does not produce negative reactions, sodium remains the only ion which can be responsible for the negative reaction to sea water. Having established that the sodium in sea water repels *Ligia*, the next step was to determine the distribution of specimens between sea water and NaCl paper. It is interesting to note that the NaCl alone proved to be six times as repellent as sea water.

TABLE I

Reaction of *Ligia* to filter paper saturated with salt solutions.

Treatment of each half of paper	Total number of isopods on each half	Number of experiments	Ratio
Normal adults			
5/8 M NaCl *vs.* sea water	32 : 205	4	1 : 6.40
5/8 M NaCl *vs.* 3.5/8 M $CaCl_2$	224 : 350	13	1 : 1.56
3.7/8 M $MgCl_2$ *vs.* distilled water	201 : 188	7	1 : 0.93
5/8 M NH_4Cl *vs.* distilled water	6 : 128	3	1 : 21.33
75% 5/8 M NH_4Cl *vs.* distilled water	1 : 54	1	1 : 54
50% 5/8 M NH_4Cl *vs.* distilled water	0 : 70	1	0 : 70
40% 5/8 M NH_4Cl *vs.* distilled water	5 : 48	1	1 : 9.60
33% 5/8 M NH_4Cl *vs.* distilled water	42 : 148	4	1 : 3.52
10% 5/8 M NH_4Cl *vs.* distilled water	19 : 132	3	1 : 7.00
5% 5/8 M NH_4Cl *vs.* distilled water	42 : 145	4	1 : 3.45
2% 5/8 M NH_4Cl *vs.* distilled water	103 : 92	4	1 : 0.89
Adults after 4 days in sea water			
Sea water *vs.* distilled water	35 : 34	1	1 : 1
Adults with antennae removed			
Sea water *vs.* distilled water	163 : 207	8	1 : 1.27
5/8 M NaCl *vs.* distilled water	52 : 127	4	1 : 2.44
Adults with spines and 7th leg removed			
Sea water *vs.* distilled water	26 : 65	2	1 : 2.50
Young just released from brood pouch			
Sea water *vs.* distilled water	102 : 192	4	1 : 1.88
5/8 M NaCl *vs.* distilled water	19 : 44	1	1 : 2.31

Former experiments (Barnes, 1939) showed that the most marked negative reaction occurs to KCl paper, the magnitude of the response being a rectilinear function of the concentration. It is known that NH_4 has physiological effects similar to K on *Ligia* (Barnes, 1935) and other invertebrates (Wells, 1928). Accordingly, the reaction to NH_4Cl paper was tested (Table I). The repellent action of NH_4 proved to be unusually strong.

It was suggested (Barnes, 1938) that stimulation of the legs by the KCl and NaCl papers forced the isopods over on the distilled water paper. This hypothesis was supported by tests showing the tendency to collect on $CaCl_2$ paper as opposed to NaCl. The former results were based on the repeated orientation of the same four isopods. Accordingly, this experiment was repeated on a large scale (Table I). The preference for $CaCl_2$ was again evident but the ratio between Ca and Na paper was lower, owing to the greater number of specimens tested.

TABLE II

Survival of *Ligia* in modified sea water.

Parts of salt solution in 100 cc. of modified sea water	Average survival	Maximum survival	Number of specimens
	hours	*hours*	
15 per cent 3.7/8 M $MgCl_2$......	80.3±8.5	144	20
33 per cent 3.7/8 M $MgCl_2$......	27.0±2.9	63	19
50 per cent 3.7/8 M $MgCl_2$......	12.9±0.6	22	18
80 per cent 3.7/8 M $MgCl_2$......	5.9±0.4	7	10
5 per cent 5/8 M NH_4Cl........	9.3±0.6	24	20
10 per cent 5/8 M NH_4Cl.......	4.1±0.4	7	10
15 per cent 5/8 M NH_4Cl.......	2.3±0.1	3	10
20 per cent 5/8 M NH_4Cl.......	1.6±0.08	2.5	20

Previous immersion in distilled water is known to prevent the negative reaction to sea water paper compared with distilled water paper (Barnes, 1939) and the present experiments (Table I) indicate that four days immersion in sea water also destroys this reaction. The purpose of these tests was to determine if the salt requirements of the animals conditioned their reactions to filter paper containing the various solutions employed.

It seemed desirable to find out what part of the animal was affected by the salt solution on the substratum. There are three possibilities: the antennae, the legs, and the uropodal spines. It will be seen in Table I that the removal of the antennae did not prevent the negative reaction to sea water. The removal of the uropods with spines together with the seventh leg eliminated the flushing mechanism to the gills. It will be recalled that water rises between the sixth and seventh legs, passes over the gills and drains down between the uropods and spines. The results presented in Table I indicate that this flushing circuit is not essential for the sea water effect.

It was reported in earlier papers that the young are released under water. Hence it became of interest to test the reactions of young isopods immediately after escape from the brood pouch. From the results (Table I) it is clear that *Ligia* begins its life history with an innate negative reaction to NaCl and to sea water. The young were tested in groups of ten on small filter papers (9 cm. in diameter).

Survival in Sea Water with Added $MgCl_2$ or NH_4Cl

The toxicity series for the common ions occurring in sea water was established as a possible means of detecting any special ionic effect on *Ligia* which might help to explain the limited survival in sea water. However, the series for single salts is the normal one for Crustacea. The next step was to determine the series for samples of sea water in which the concentration of one ion was progressively increased. This afforded more natural conditions. The graph published in the preceding paper did not include $MgCl_2$ and NH_4Cl. The present experiments (Table II and Fig. 1) were performed to complete this series. It is ap-

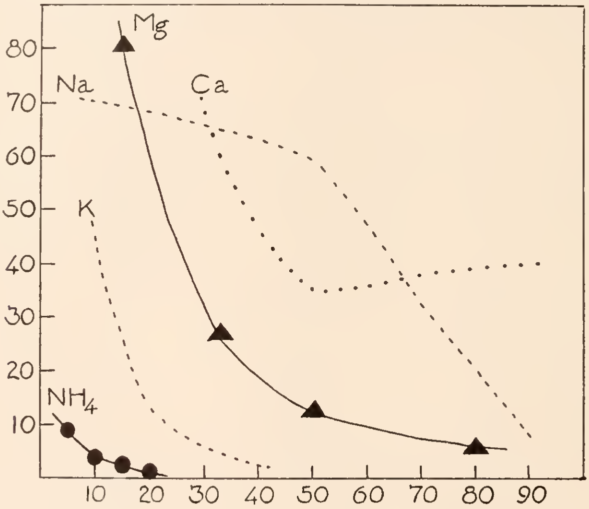

FIG. 1. Survival times of isopods in sea water in which the concentration of one ion is increased. Ordinates: average survival in hours. Abscissae: concentration of 5/8 M KCl, NaCl, and NH_4Cl and of 3.5/8 M $CaCl_2$ and 3.7/8 M $MgCl_2$ in cc. per 100 cc. of modified sea water. Triangles: $MgCl_2$; solid circles: NH_4Cl. The curves for KCl, NaCl, and $CaCl_2$ previously determined (Barnes, 1939) are indicated as dotted lines for comparison with the new data on $MgCl_2$ and NH_4Cl. Note the severe toxicity of NH_4Cl.

parent from the data that these two ions, when tested in sea water, show the same relative toxicity as the single salts.

Effect of Oxygen in Diluted Sea Water

Earlier experiments indicated a limited survival of only about eighteen hours in 50 per cent sea water, but it was noted that the frequency of the gill beat increased. This suggested that continuous oxygenation of the diluted sea water might increase the longevity. Table III indicates that the survival in 50 per cent sea water is increased threefold by bubbling oxygen through the solution. The isopods were tested singly in finger bowls containing 100 cc. of solution. The level of the solution was marked on the bowl and distilled water was added from time to time to compensate for evaporation. Oxygenation had little effect on the survival in 25 per cent sea water. This was similar to previous results with single salts (Barnes, 1939).

TABLE III

Survival of *Ligia* in natural sea water and dilutions.

Conditions	Average survival	Maximum survival	Number of specimens
	hours	*hours*	
Young just released from brood pouch. Each in 20 cc. of sea water	121.8± 4.9	148	14
Adults. Each in 1000 cc. of sea water	85.1±16.6	195	10
Adults. Each in 100 cc. of oxygenated 25% sea water	11.6± 0.4	16	16
Adults. Each in 100 cc. of oxygenated 50% sea water	60.2±11.5	96	10

Survival of Young Ligia in Natural Sea Water

It was reported (Barnes, 1935) that only immature specimens have been found in the sea and that the very young isopods are too small to migrate landward with the large specimens when the tide rises. These observations are now correlated with the immersion tests reported in Table III showing that recently released young survive for an average of 122 hours in sea water compared with 85 hours for adult controls tested at the same time. The mature specimens which served as controls were each immersed in 1 liter of sea water to compensate for the small size of the young (2.5 mm.), each in 20 cc. of sea water.

Discussion

The significance of the experimental results is indicated in the preceding sections of this paper to relate the new data to previous reports. The evidence is now fairly complete that sodium is responsible for the negative reaction to filter paper containing sea water. This result was rather unexpected as NaCl is the least toxic of the common cations for *Ligia*. The reactions of the isopods to substrata containing salts is undoubtedly modified by their previous history. Immersion in distilled water or sea water abolishes the preference for distilled water paper compared with sea water paper. Bateman (1933) showed that salts are concentrated by evaporation in specimens in air and as Miller (1938) points out, lost water must be replaced. This may be a factor in the collection of animals on the distilled water side of the filter paper. However, the actual flushing of the gills is not necessary for this reaction as the ablation experiments show. Nor are the antennae essential although the aversion for sea water is not so marked in specimens with antennae removed. This is seen in an experiment not listed in Table I. Isopods with antennae removed and others with uropods and seventh leg removed were allowed to orient in the same dish on filter paper containing sea water on one half and distilled water on the other. The ratios for sea water vs. distilled water were: for antennaless specimens, 21:45; for spineless specimens, 8:60.

Both the graphs for sea water containing additional Mg and NH_4 indicate severe toxic action not unlike the effect of K. The anaesthetizing action of $MgCl_2$ was evident at a concentration of $\frac{1}{8}$ M in sea water. These modifications of sea water in which the concentration of one ion is increased are of speculative interest in connection with the geo-chemistry of the sea. Thus the same amount of Na and K have been supplied to the sea from primary rocks, i.e. 16.8 and 15.0 grams per kg. of water, but at present these quantities are 10.7 and 0.37 grams per kilo. The low K content of the sea is ascribed to the very great adsorption of its ions on the finely divided hydrolysate sediments (cf. Goldschmidt, 1937).

The prolonged survival of *Ligia* in oxygenated 50 per cent sea water might be expected from the increased frequency of gill beat in diluted sea water. The increased respiration probably supplies the additional energy expended in resisting osmotic disturbances in hypotonic media (for references, cf. Barnes, 1934).

It is now clear that *Ligia* begins its life history in the sea. Of hundreds of " births " observed only one or two occurred out of sea water or solutions. Moreover, the young survive immersion longer than adults, although the aversion for sea water and NaCl on filter paper is evident from the moment they are released from the brood pouch.

Summary

1. Filter paper moistened with ⅝ M NaCl is approximately six times as repellent to *Ligia* as sea water paper.

2. An equal distribution of isopods occurs between filter papers containing 3.7/8 M $MgCl_2$ and distilled water.

3. Of all ions tested, the most pronounced aversion is shown for NH_4Cl paper (in concentrations of $\frac{1}{30}$ M and above).

4. The tendency of *Ligia* to collect on a filter paper containing distilled water when the other paper contains sea water is not prevented by the removal of the antennae or by removal of the mechanism for flushing the gills (seventh legs and uropods).

5. Young isopods immediately after release from the brood pouch have a marked aversion for filter paper containing ⅝ M NaCl or sea water. Their tolerance for immersion in sea water is greater than that of adults.

6. The distribution of *Ligia* on filter paper containing distilled water, sea water, or other salt solutions is probably determined by two factors: (*a*) the salt requirements of the organism and (*b*) the stimulating effect of salt on the leg movements.

7. The survival of *Ligia* in 50 per cent sea water is prolonged by oxygenation.

The writer is indebted to Dr. J. F. G. Wheeler for many courtesies.

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DIURNAL CHANGES IN THE ELECTRICAL RESPONSE OF THE COMPOUND EYE[1]

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Diurnal changes in the physical environment have resulted in the establishment of biological diurnal rhythms in a large number of organisms. Many of these biological rhythms are apparently under the direct control of the environment for they disappear as soon as the organism is subjected to a constant environment. Others, however, appear to be inherent rhythms for they continue for long periods of time after the organism is placed in a constant environment. Welsh (1938), in his review of the rather extensive literature on this subject, has cited numerous examples of persistent diurnal rhythms and has discussed the possible mechanisms that might be involved in the control of such rhythms.

While engaged in a study of the electrical potentials associated with illumination of the compound eye of certain arthropods, the authors made the interesting observation that the magnitude and form of the electrical response from the eye of the carabid beetle, *Chlaenius diffinis,* vary according to the time of day, even though the animal was kept continuously in the dark. Further investigation disclosed that such daily changes in the potentials from the eyes occurred in a number of other beetles. It is the purpose of this report to describe these diurnal fluctuations in potential and to correlate them with other types of diurnal changes that are known to occur in the eyes of certain invertebrates.

Materials and Methods

For this investigation the following beetles were employed: *Chlaenius diffinis, Chlaenius tomentosus, Hydrus triangularis, Harpalus pennsylvanicus, Harpalus caliginosus, Anomoglossus emarginatis, Osmoderma eremicola,* and *Necrophorus orbicollis.*[2] A number of experiments were also performed with the crayfish, *Cambarus virilis.* All these animals were obtained directly from the field and, with few exceptions, were

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[1] Aided by a grant from the Rockefeller Foundation for work in Cellular Physiology.

[2] The authors are indebted to Prof. H. E. Jaques of Iowa Wesleyan College for the identification of these beetles.

used within a few days after being brought into the laboratory. The experiments were all performed between May and October, 1939.

The method of obtaining and recording the potentials from the eye is similar to that described in connection with studies on the grasshopper (Jahn and Crescitelli, 1938). The method consists in leading off from the corneal surface of one eye by means of a silver-silver chloride electrode. The electrode dips into a reservoir of physiological solution which, in turn, is connected by means of a small glass siphon to a solution-filled chamber built around the eye. The front of this eye-chamber is transparent and permits illumination of the eye. The other eye is prepared in the same manner except that the front of the chamber is opaque. This permits a study of the potential which develops across the two eyes when one eye is illuminated. The intensity of the light striking the eye and the exposure time is controlled and known in all cases. The potential, after amplification through a condenser-coupled amplifier (time constant of 1.9 seconds), is recorded by means of a cathode ray oscillograph and camera.

To demonstrate the occurrence of a diurnal rhythm in the electrical response, the animal, prepared in the above manner, is placed in a darkened moist chamber and kept there throughout the course of the experiment. At intervals of an hour or two throughout the day and evening a flash of bright light (725 foot candles) is admitted to the eye and the resulting deflection of the cathode ray beam is photographed. In some animals it is possible to continue the experiment for 3–4 days; in others, however, because of the limited life of the animal under the conditions of the experiment, only a 24-hour run is possible. Temperatures were recorded with a resistance thermometer throughout a number of the experiments, and it was found that either the temperature remained approximately constant or that the variations did not coincide with variations in the electrograms. Reference to barograms which were recorded during the period of the present experiments showed that small diurnal changes in barometric pressure did tend to occur but that this tendency was very often masked by much larger progressive variations which had no relation to the diurnal cycle.

General Description of the Electrical Response of the Arthropod Eye

The form and magnitude of the electrical response which is obtained by illumination of the compound eye of arthropods varies with the intensity of illumination, the length of the exposure period, the degree of light- or dark-adaptation of the eye, and the particular species of animal that is involved (Jahn and Crescitelli, 1938; 1939). For the grasshopper (*Melanoplus differentialis*) the electrical response of the dark-

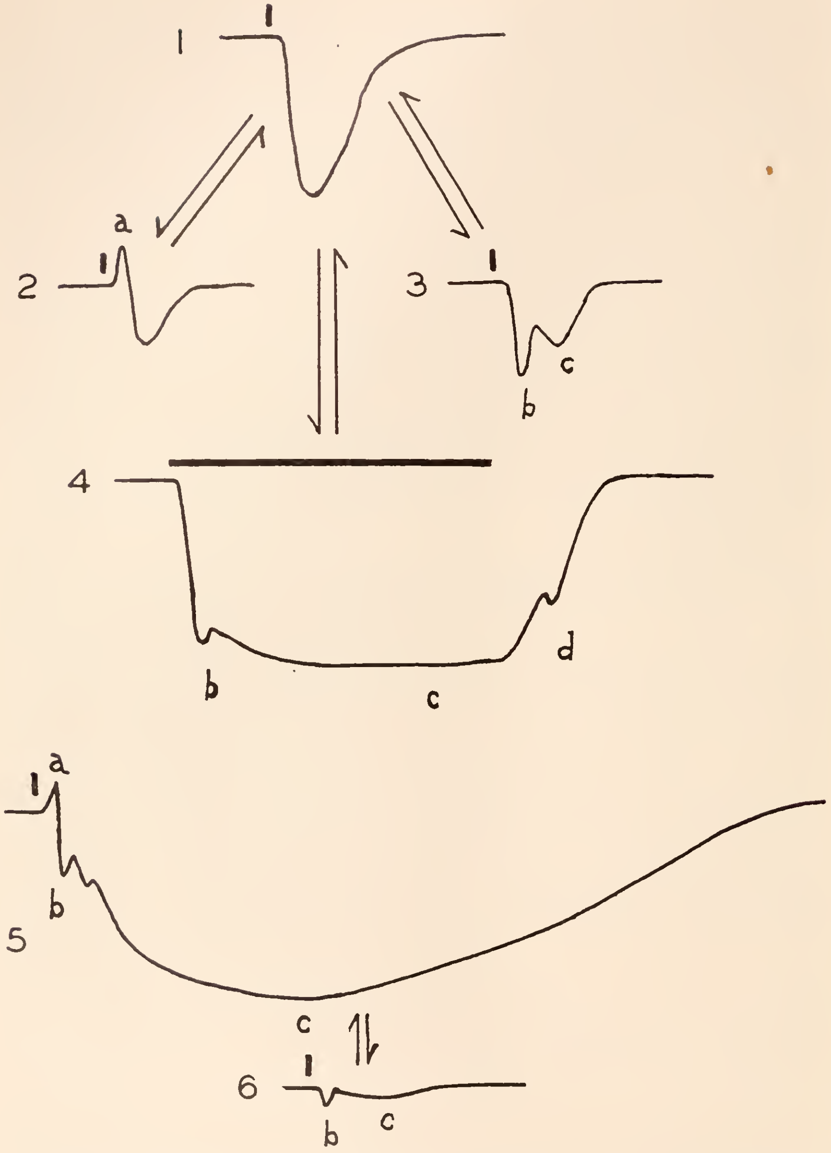

FIG. 1. Diagrams of typical electrical responses. Record 1, dark-adapted grasshopper, brief flash of high intensity; record 2, dark-adapted grasshopper, brief flash, low intensity; record 3, light-adapted grasshopper, high intensity, brief exposure; record 4, light-adapted grasshopper, high intensity, one second exposure; record 5, dark-adapted Cecropia moth, high intensity, brief exposure; record 6, light-adapted Cecropia moth, high intensity, brief exposure.

adapted eye to a brief exposure of bright light is of a relatively simple type (Record 1, Fig. 1). It consists of a rapid deflection (downward) indicating the development of negativity at the illuminated eye with respect to the non-illuminated eye. Following this is a relatively slow decline of the potential. This wave form is somewhat more complex when the intensity of the stimulating light is low (Record 2, Fig. 1) or when the eye is light-adapted (Record 3, Fig. 1). In the Cecropia moth (*Samia cecropia*) the typical response of the dark-adapted eye to a brief exposure of bright light is that shown in Record 5, Fig. 1. This response is obviously polyphasic, consisting of a number of elements, the most typical being the a-, b-, and c-waves. The a-wave is a small, spike-like deflection which indicates a condition of positivity of the illuminated eye with relation to the other eye. Following the a-wave is the rapid negative deflection known as the b-wave while the c-wave is the very slow and prominent terminal phase. In addition to these three elements there may be several poorly characterized deflections located between the b- and c-waves. Light-adaptation of the eye results in abolishment of the a-wave, a reduction in magnitude of the b-wave, and a reduction in both magnitude and duration of the c-wave (Record 6, Fig. 1). Electrograms similar to those obtained from the Cecropia moth have also been noted in the Luna moth (*Actias luna*), the Promethea moth (*Callosamia promethea*), and other moths. The electrical responses of the grasshopper and Cecropia moth eyes have been studied between the hours of 7:00 A.M. and midnight on numerous occasions, and in no case was any evidence obtained that the magnitude and wave form of the response varied with the hour of the day.

Results and Discussion

Evidence of a Diurnal Rhythm in Beetles

Diurnal fluctuations in the electrical response have been obtained with the following beetles: *Chlaenius diffinis* (6), *Chlaenius tomentosus* (1), *Harpalus pennsylvanicus* (2), *Harpalus caliginosus* (1), and *Hydrus triangularis* (3). The numbers in parentheses indicate the number of animals of each species that were used. Records 1–30, inclusive, Fig. 2, demonstrate the diurnal changes as they occur typically in *Chlaenius diffinis.* This animal was studied for four consecutive days. (The records obtained on the first day, which are identical with those of the second day, are not included in this group because they are used later (Records 67–77, Fig. 3) in the discussion of another group of results.) Record 1, which is the response to a brief exposure, was photographed at 9:20 A.M. of the second day. The response is fairly simple in nature and not greatly unlike that obtained from the grasshopper eye. The same type of response was elicited from the eye during the morning and early afternoon hours (Records 2–5). At 3:57 P.M. (Record 6) a distinctly different type of electrogram was obtained. The wave form of this record is polyphasic, consisting of an initial series of relatively rapid waves and a long slow phase (c-wave). From 3:57 P.M. to 11:02 P.M. (Records 6–11) this complex wave form persisted, and the slow wave increased in magnitude. On the morning of the third day of the experiment (Record 12) a brief exposure of the eye to light again elicited the relatively simple type of electrogram. This persisted with slight modifications until 5:11 P.M. (Records 13–19). At 6:33 P.M.

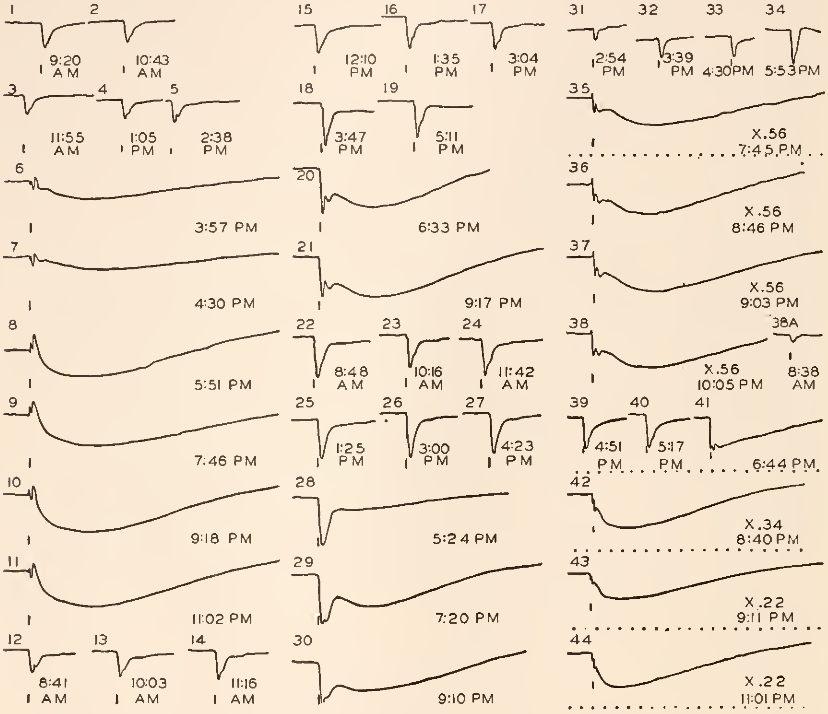

FIG. 2. Electrical responses from the eyes of certain beetles at different hours during the day and evening. All responses are to brief exposures of a bright (725 f.c.) light. The records shown are typical responses selected from many which comprised each series. All records of Figs. 2 and 3 were taken with the recording paper moving at the same speed. Distances between dots of the time record, when shown, indicate 142 sigma. The records for each animal were taken with the same amplifier gain except when indicated as X. These and the following records have been retouched to facilitate reproduction. Refer to text for further details.

of the third day (Record 20) the relatively complex wave form was again obtained although in this case it was somewhat different from the polyphasic wave of the first and second evenings, indicating a progressive change with time. The simple wave form was once more recorded on the morning of the fourth day (Record 22) and persisted throughout that day (Records 23–27). At 5:24 P.M. (Record 28) a brief exposure to light called forth a response showing the beginning of a slow wave, and at 7:20 P.M. (Record 29) and 9:10 P.M. (Record 30) polyphasic electrograms were again recorded.

The diurnal rhythm in another specimen of *Chlaenius diffinis* is presented in Records 31–38A (Fig. 2), inclusive. During the afternoon (Records 31–34) the responses obtained from this animal were of the typically simple type. During the evening hours (Records 35–38) a complex type of wave form, not greatly unlike that obtained from certain moths, was recorded. These electrograms possess the a-, b-, and c-waves described in connection with the Cecropia moth (Fig. 1). The next day (Record 38A) the typical day-type of response was once again obtained. A series of records from still another specimen of *Chlaenius diffinis* are shown in Fig. 3 (Records 85–96, inclusive). In this case the complete change from the night-type to the day-type did not occur until the late morning hours of the second day. Since the animal was found dead late in the afternoon, it seems as if the delay in the change of the response to the day-type might have been associated with the degenerative changes occurring in the animal.

The electrical responses from the eye of the water beetle, *Hydrus triangularis,* also undergo marked diurnal variations in both form and magnitude (Records 60–66, 97–107; Fig. 3). In many respects the diurnal changes that occur in this beetle are not greatly unlike those that occur in *Chlaenius diffinis.* The day-type of response is here also relatively simple while the night-type of response is polyphasic and contains the characteristic slow c-wave.

A diurnal change in the potential from the eye occurs also in the carabid beetle, *Chlaenius tomentosus* (Records 45–59, inclusive, Fig. 3). Here also the day-type of response (48–51, 59) is relatively simple and lacking in any distinct slow component. The night-type of response is again larger in magnitude, polyphasic, and characterized by the presence of a definite slow wave. Incidentally, it may be noted that the night-type of response which was obtained during the first evening (45–47) is different from the response which was photographed during the second evening (52–58), indicating a change with time.

In two other species of carabid beetles, *Harpalus caliginosus* and *Harpalus pennsylvanicus,* it has been found that a rapid and relatively

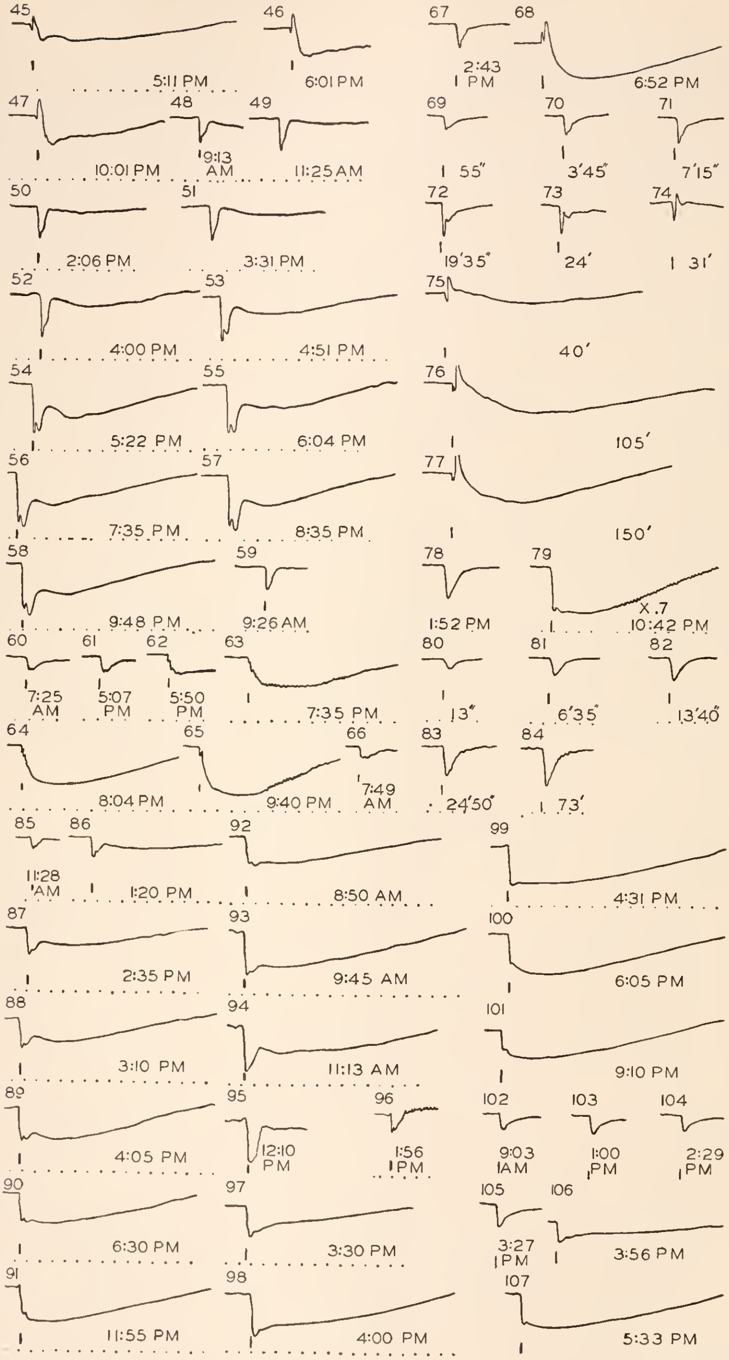

FIG. 3. Electrical responses from the eyes of certain beetles at different hours of the day and evening (Records 45–66, 85–107) and the effect of light adaptation on the response (Records 67–84). For further details refer to text.

simple type of response is obtained during the morning and afternoon hours, whereas a much larger and relatively complex type of electrogram is characteristic of the evening hours. Records 39–44 (Fig. 2) illustrate these changes for *Harpalus caliginosus.*

It is clear from these results that a diurnal variation exists in the electrical response from the eyes of a number of species of beetles when the animals are kept in total darkness. There is a characteristic day-type of response present in all the beetles studied which is significantly different in form and magnitude from the typical night-type of response. The transformation from the day-type to the night-type of response occurs generally between 5:00 P.M. and 7:00 P.M. although in a number of cases the beginning of the change occurred as early as 3:30 P.M. The exact time at which the change occurs from the night-type to the day-type of electrogram has not been determined. In one beetle (*Chlaenius diffinis*) the change took place between 4:00 A.M. and 7:00 A.M. Not enough all-night studies have been made of this point, however, to warrant any conclusions.

A few experiments have been made on beetles which, apparently, do not possess a diurnal rhythm of the type described here. In one experiment on *Anomoglossus emarginatus,* one experiment on *Osmoderma eremicola,* and one experiment on *Necrophorus orbicollis,* no indications of diurnal fluctuations in the electrical potentials were obtained. This paper is not intended to be a survey of the beetles, however, and many more species will have to be examined before any generalizations and conclusions may be made regarding the significance of the presence or absence of a diurnal rhythm.

Effect of Light-Adaptation

If the dark-adapted eye of a beetle which is in the night-phase of its rhythm is light-adapted, the magnitude of the response to a single brief exposure is reduced markedly and the form of the response is altered. If this eye is then allowed to dark-adapt, the original magnitude and wave form slowly return. These facts are illustrated for *Chlaenius diffinis* in Records 67–77 (Fig. 3). In this series Records 67 and 68 show respectively the day-type and night-type of responses of the dark-adapted eye taken respectively at 2:43 P.M. and at 6:52 P.M. of the same day. At 7:00 P.M. the eye, when in the night-phase of its cycle, was light-adapted by exposure to a 725 foot-candle light for 15 minutes. Record 69, which was made 55 seconds after cessation of light-adaptation, shows the response of this light-adapted eye to a single brief exposure. The wave form has changed and the magnitude of the potential has been reduced. Records 70–77 show the gradual restoration of the night-type of response. The restoration is rather slow for even after 150 minutes of dark-adaptation (Record 77) the original magnitude and form of Record 68 had not been restored. (The electrograms which were obtained the following night (Records 8–11, Fig. 2) were almost identical in wave form with that of Record 68, Fig. 3.)

Light-adaptation of a dark-adapted eye which is in the day-phase of its cycle results in a reduction in magnitude but not in an appreciable alteration of wave form. Records 78–84 demonstrate this for *Hydrus triangularis*. In this series the day-type and night-type of responses were recorded respectively at 1:52 P.M. (Record 78) and at 10:42 P.M. (Record 79) of the same day. At 2:40 P.M. the eye, when still in the day-phase of its cycle, was light-adapted and the response (Record 80) taken 13 seconds after cessation of light-adaptation, illustrates the effect of the light-adaptation. Recovery from light-adaptation is shown in Records 81–84. Seventy-three minutes after cessation of light-adaptation (Record 84) the original magnitude of the dark-adapted eye had been restored.

Interpretation and Conclusions

Although a considerable number of investigations have appeared on the electrical phenomena of the vertebrate and invertebrate eyes (for literature refer to Granit, 1936; Jahn and Crescitelli, 1938, 1939), this appears to be the first report of a diurnal rhythm in the electrical response of the eye. Since the origin of the potentials in the eye is not known, it is impossible to deduce from the potentials alone the nature of the diurnal changes occurring in the eye that are responsible for the diurnal changes in the electrical responses. There are a number of already well established facts, however, that may be correlated with the diurnal variations in electrical potentials. It has been known for some time that persisting diurnal changes in the position of the distal and proximal pigments occur in the eyes of certain arthropods (Welsh, 1938). Such rhythmic changes in the position of the pigment have not been observed in any of the beetles studied, but it is possible that such changes do occur and may be related to the observed diurnal variations in the electrical response. It is known that the form and magnitude of the response is modified by light- and dark-adaptation of the eye (Jahn and Crescitelli, 1938, 1939) as is also the position of the proximal and distal pigments (Parker, 1932). One way of resolving this question would be to study the electrical responses in an animal in whose eyes are definitely known to occur daily rhythmic migrations of the pigment. The crayfish (*Cambarus virilis*) is one in which the position of the proximal pigment is known to vary with the time of day (Bennitt, 1932). We have experimented with a number of freshly caught crayfish whose eyes showed a persisting diurnal rhythm in glow, but in no case were we able to obtain definite evidence of a diurnal rhythm in the electrical response. Studies which are in progress on the morphology of some of the beetle eyes used in this investigation may be of some assistance in reaching a tentative conclusion regarding the relationship between the character of the response and the position of the pigment.

The mechanism which is in primary control of these diurnal periodic changes is not known. The suggestion has often been made of the existence of an inherent physiological process operating independently of the environment and acting as a timing mechanism (Welsh, 1938). In the present experiments a number of environmental factors can be dismissed as controlling factors. These are illumination, moisture, temperature and barometric pressure, all of which were either controlled or eliminated as explained under "Materials and Methods." Ionization of the air has apparently been eliminated by Horstmann (1935) as a determining influence in the diurnal migration of the eye pigment in certain moths. The possibility that cosmic radiation may be the controlling factor is rendered unlikely since Millikan (1932) found little, if any, difference in the intensity of this radiation at different times during the day. There is always the possibility that some other environmental influence, as yet unsuspected, may serve as the stimulus for the electrical rhythm that has been observed. Until such an influence is found, however, we must conclude that an internal mechanism acting independently of the immediate environment lies at the basis of the electrical rhythm.

SUMMARY

1. A diurnal rhythm occurs in the electrical responses obtained from the compound eyes of certain beetles (*Chlaenius diffinis, Chlaenius tomentosus, Hydrus triangularis, Harpalus caliginosus, Harpalus pennsylvanicus*). When one of these beetles is kept in total darkness and under approximately constant environmental conditions, the electrical response to a brief exposure of light which is recorded during the morning and afternoon hours (day-type) is markedly different from the response obtained during the late afternoon and evening hours (night-type).

2. The day-type of record is relatively simple and not unlike that which is always obtained from certain grasshoppers and butterflies. The night-type of response, always of greater magnitude, possesses a complex wave form similar in many respects to that always elicited from the eyes of certain moths.

3. The day-type of response is reduced in magnitude but not greatly altered in form when the eye is light-adapted. The night-type of electrogram is not only reduced in magnitude by light-adaptation of the eye, but the wave form is markedly altered.

4. The possibility is suggested that the diurnal cycle in the electrical response may be related to a diurnal migration of eye pigments.

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THE DIGESTIVE TRACT OF CARASSIUS AURATUS

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INTRODUCTION

There have been many contributions, mainly morphological, to the knowledge of the structure of the alimentary tract of fishes. The most recent work[1] has been done by Babkin and Bowie (1928), Dawes (1929), Blake (1930, 1936) and Rogick (1931). An extensive review of the earlier literature is given by Dawes (1929) as well as by Blake (1930) and Rogick (1931).

While investigating the function of the alimentary canal in *Fundulus heteroclitus,* Babkin and Bowie (1928) found that it possessed no stomach nor any gland for the secretion of pepsin and hydrochloric acid. Accordingly, they studied the anatomy as well as the physiology of the tract in this species. They found that the first portion of the intestine joined directly to the oesophagus and was capable of dilating to form a container for food. Since the bile and pancreatic ducts entered this region of the gut, it was designated the duodenum. A sphincter between the oesophagus and intestine closely resembled in structure the pyloric sphincter of higher vertebrates.

In preparation for a physiological study of digestion in the plaice, Dawes (1929) investigated the histological aspects of its digestive tract. This species possesses a stomach with true digestive glands. The walls of all parts of the tract showed a similar fundamental plan in possessing mucous membrane, sub-mucous coat, inner circular and outer longitudinal muscle layers and a serosa. There was no evidence of either muscularis mucosae, stratum granulosum or stratum compactum. Consequently, the important variations in structure occurred in the mucous membrane and muscular layers of the separate regions. These were studied in considerable detail.

Blake, in 1930, presented a histological study of the gut of the sea-bass, *Centropristes striatus.* This fish, a predaceous teleost, was selected because of its feeding habits. For purposes of comparison, Blake studied later (1936) the digestive tract of the sea robin, *Prionotus carolinus,* a bottom-feeding form. He found the fundamental structure of the gut to be similar in both species. The oesophagus was a short tube leading into an elongated stomach. Pyloric caecae opened into the anterior end of the intestine. A distinct valve separated the intestine and rectum in *Centropristes* but could not be demonstrated in *Prionotus.*

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[1] Curry, E. 1939. The histology of the digestive tube of the carp (*Cyprinus carpio communis*). Abstract was received after completion of the work on *Carassius.*

Rogick (1931) investigated the microscopical anatomy of the entire tract of the minnow, *Campostoma anomalum* (Rafinesque). She found that the pharynx could be divided into an anterior and posterior region on the basis of characteristic structures present in each. The anterior part contained the visceral clefts. In the posterior portion were ventral pharyngeal teeth and a dorsal callous pad. A dorsal pocket and pneumatic duct characterized the short oesophagus. As in *Fundulus,* an enlargement of the intestine, the intestinal bulb, served as a container for food and represented that part of the gut normally occupied by the stomach. No gastric glands were found in this region. The intestine proper was of very simple type, lined with columnar epithelium and goblet cells.

The present paper includes a study of the gross and microscopical anatomy of the digestive tract of the goldfish, *Carassius auratus.* The investigation was begun at Yankton College at the suggestion of Dr. Austin P. Larrabee. At this time, it was found that *Carassius,* like *Fundulus* and *Campostoma,* does not possess a true stomach. It seemed desirable, therefore, to make a thorough histological study of the entire tract. In view of the absence of the stomach, it was of interest to determine whether glands of the gastric type could be found in any other part of the digestive tube. In this connection, the nature of the cells in the intestinal epithelium was also of importance. Consequently, the gross and microscopical anatomy of the posterior pharynx, oesophagus and intestine have been studied in detail.

Material and Methods

Both domesticated and wild goldfish were used. Of a total of sixteen individuals, four were studied for gross structure and twelve for microscopic. Both Helly's and Bouin's fixatives were employed. Transverse and longitudinal sections were made of the anterior portion of the tract. The intestine was cut transversely. Sections were mounted serially and successive slides were stained with Delafield's haematoxylin and eosin, Mallory's triple connective tissue stain, Heidenhain's iron alum haematoxylin and eosin, and mucicarmine.

Gross Anatomy

Table I shows the measurements for eight individuals. Total lengths included the distance from the tip of the snout to the end of the caudal fin. Measurements of the digestive tract as a whole were taken from the beginning of the posterior pharynx to the anus. It appears from these figures that the rather wide individual variations in length of the tract result chiefly from variations in length of the intestinal portion. In all cases, the digestive tube was considerably longer than the body cavity.

The posterior pharynx extended from the last gill arch to the beginning of the oesophagus. It appeared to be a more or less rigid organ; the bones of the ventral and lateral walls as well as the dorsal horny pad permitted very little stretching. It was wider at the anterior end and narrowed gradually to its junction with the oesophagus. The anterior part did not lie free in the body cavity but was connected with the head musculature.

Table I

Showing measurements of the body and of parts of the digestive tract in centimeters

Animal	Total length	Length of body cavity	Total length of tract	Posterior pharynx	Oesophagus	Intestine
A	12.0	5.0	40.0	0.8	0.3	38.9
B	13.0	4.5	19.0	0.5	0.2	18.3
D	12.0	4.5	29.95	0.6	0.25	29.1
E	15.0	5.0	35.0	0.7	0.3	34.0
F	12.0	4.0	30.5	0.6	0.3	29.6
H	11.0	4.0	23.45	0.6	0.25	22.6
I	13.5	4.8	32.0	0.8	0.3	30.9
J	12.6	4.5	29.92	0.65	0.27	29.0

This portion of the pharynx contained the pharyngeal teeth. They were located laterally on a more or less horizontal line and slanted in a slightly dorsal direction. There were usually four teeth on each side attached to the inferior pharyngeal bones. The angle of their insertion permitted them to bite on the dorsal horny pad. The teeth were narrow, conical and lay with their flat sides closely against each other. The first tooth on each side was firmly joined to the bone while the rest of the teeth were more loosely attached. At the base of each tooth, and apparently attached to it where it joined the inferior pharyngeal bone, was an immature tooth.

The posterior pharynx was characterized also by the dorsal horny pad which lay at the junction of the two parts of the pharynx. It was roughly triangular in shape, the wide end directed anteriorly. It had a median longitudinal ridge and, as a result, presented a more convenient biting surface for the teeth.

The oesophagus was a very short tube, averaging 0.3 cm. in length and 0.2 cm. in width. Both the anterior and posterior boundaries were clearly marked. From the dorsal surface extended a small diverticulum which connected with the second chamber of the air bladder.

The junction of oesophagus and intestine was marked by a circular constriction, the intestinal sphincter, and also by the sudden enlargement of the intestine to form the intestinal bulb. Although there was no sharp distinction, histologically, between this part and the intestine proper, a difference was evident both in relative size and in the complexity of the mucosal folds. The intestinal bulb was larger at its anterior end, quite straight, and narrowed gradually until it merged into the intestine proper. At its widest point its diameter was two or three times that of the rest of the intestine. The intestinal bulb was capable of great expansion. This was particularly evident in fish examined just after eating when the bulb was extended to about three times its original size. At such a time, the walls were so thin that the herringbone pattern of the mucosal folds could be clearly seen.

The intestine proper had a much smaller diameter than the intestinal bulb and was not capable of such great distension. The width was more or less uniform throughout, becoming slightly smaller as the anus was approached. The rectum, or terminal portion, was distinguished from the rest of the intestine by its straight course to the anus.

The intestine as a whole was greatly coiled. It was entirely separate from the air bladder and occupied the ventral portion of the body cavity. The method and direction of coiling followed the same general plan in all the fish studied, although individual details might differ. The first limb of the intestine, including the intestinal bulb and a small part of the intestine proper, extended without coiling to the posterior end of the body cavity. There, it turned sharply and continued anteriorly, usually toward the left side. From this point, the coiling showed individual variation. In general, however, it passed from the left to the right side and most of the coils were found on the right side. At some point in these coils on the right side, the intestine turned on itself and retraced the previous coilings. This continued until the terminal portion straightened out and became the rectum. When the intestine was uncoiled, this sharp bend appeared approximately in the middle of its length. The last part of the intestine was somewhat smaller and darkened with fecal matter but was, otherwise, not differentiated.

Microscopic Anatomy

Pharynx

The mucous membrane of the posterior pharynx formed rather low, convoluted and branching folds. At the more anterior end, there appeared to be no regularity in arrangement but, toward the caudal end, these irregular folds became somewhat organized and continued into the longitudinal folds of the oesophagus. On the ventral surface, the folds were uniform in height producing the plateau-like appearance which Rogick (1931) found in the minnow. Near the pharyngeal teeth they were greatly elongated.

The wall of the pharynx consisted of three layers, the tunica mucosa composed of epithelium and sub-epithelial connective tissue, the tunica muscularis and the tunica serosa. These same three layers were found also throughout the rest of the tract. Absence of a muscularis mucosae in the digestive tract of fishes has led to certain departures from the commonly accepted terminology. Bridge (1910), in the Cambridge Natural History, designated the sub-epithelial tissue as submucosa; the mucosa, accordingly, comprised only the epithelium and basement membrane. Rogick (1931) followed this method in her description of the minnow. Blake (1930, 1936) and Scott and Kendall (1935), however, distinguished a tunica propria immediately below the epithelium which continued without interruption into the submucosa. Since this follows more closely the interpretation of comparable tissues in the higher vertebrates (Maximow-Bloom, 1938), the term " tunica propria " will be used throughout the present paper to indicate the connective tissue lying directly beneath the epithelium and forming the core of the mucosal folds.

The pharyngeal epithelium was apparently stratified columnar of a rather specialized type. The surface cells were greatly elongated mucous cells, closely packed, and resting on several layers of undifferentiated polyhedral cells (Fig. 1). These goblet cells were not so numerous at the tips of the mucosal folds and, in some cases, were entirely absent. They stained red with mucicarmine and showed a large-meshed cytoplasmic reticulum within the body of the cell.

A basement membrane could be distinguished although it was sometimes obscured by the dense connective tissue fibers immediately below it. These closely packed fibers stained intensely with Mallory's stain and were considered to be the stratum compactum. They formed a distinct layer in the posterior pharynx and could be identified in other parts of the tract as well. Rogick (1931) described a hyaline stratum compactum in the anterior pharynx but found no comparable structure in the posterior pharynx, oesophagus or intestine of the minnow. A

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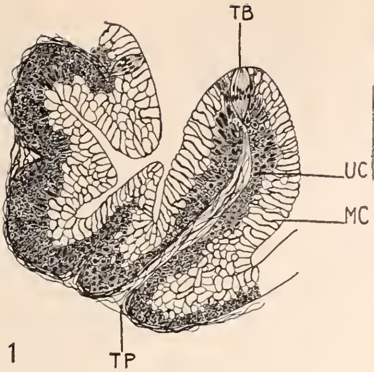

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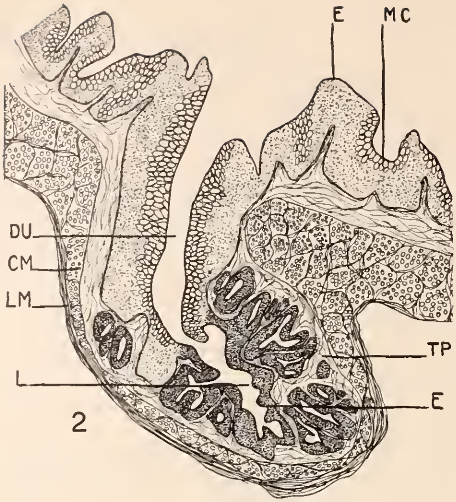

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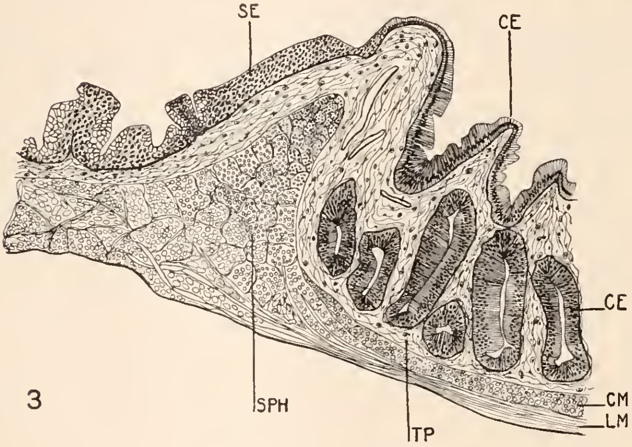


Abbreviations in Figures

BC—Basophilic (mast) cells
CE—Columnar epithelium
CM—Circular muscle
DU—Duct
E—Epithelium
L—Lumen
LD—Liver duct
LM—Longitudinal muscle
M—Mesentery
MC—Mucous cells
N—Cells of myenteric plexus
P—Pancreas
PD—Pancreatic duct
S—Serosa
SC—Stratum compactum
SE—Stratified epithelium
SPH—Sphincter
TB—Taste bud
TP—Tunica propria
TPL—Top plate
UC—Undifferentiated cells
V—Vacuoles

Fig. 1. Longitudinal section of the mucosa of the posterior pharynx showing the stratified epithelium with mucous cells. Two taste buds are shown. × 80.

Fig. 2. Semi-diagrammatic longitudinal section of the oesophageal diverticulum, showing its relation to the oesophagus. × 40.

Fig. 3. Longitudinal section through the intestinal sphincter showing the transition from stratified epithelium of the oesophagus to simple columnar of the intestinal bulb. The sphincter marks the termination of the circular muscle of the oesophagus and the origin of the circular and longitudinal muscles of the intestine. × 40.

layer of dense fibers which she found in the mouth and which she likewise considered the stratum compactum appeared similar in every way to the layer of dense connective tissue which was found throughout the digestive tract of *Carassius* and which Bolton (1933) observed in the trout and salmon.

Taste buds were present in the epithelium of the posterior pharynx. They were elongated and rather narrow. The cells extended from the basement membrane to a point just below the surface of the epithelium (Fig. 1).

The tunica propria of the pharynx contained many striated muscle fibers. Although they were chiefly longitudinal, some circular fibers were interwoven among them and they extended into the folds of the mucosa. Bolton (1933) found mast cells in the tunica propria of the digestive tract in several species of trout and salmon. His observations have been confirmed for *Carassius auratus* in the material which was fixed in Helly's solution.

There was no very sharp distinction between the tunica propria and tunica muscularis because of the muscle fibers which invaded the tunica propria. The muscular layer, itself, consisted of very heavy, striated circular fibers which were continuous with the circular muscle of the oesophagus.

Since the pharynx did not lie free in the body cavity, there was a serosa over the most posterior part only. It possessed the usual constituents, a thin layer of fibrous tissue covered by simple pavement epithelium.

Only a few imperfect sections of the functional pharyngeal teeth were obtained but their relation to the inferior pharyngeal bones could be determined. Longitudinal sections of the developing teeth presented much the same picture as that found in mammals. The primordium occupied a position in the thick muscular coat of the pharynx and was surrounded by connective tissue which was essentially the same as that of the tunica propria. The dentine was widest at the tip of the papilla and tapered down the sides to a very narrow line. It showed a faint but definite striation. On the outside of the dentine, closely applied to it, was a single layer of enamel cells. The odontoblasts, on the inner side of the dentine, were not so clearly defined as the enamel cells. The inner ends of the odontoblasts merged with the dental pulp.

Oesophagus

The tunica mucosa formed ten or twelve longitudinal folds which extended the length of the oesophagus. They were generally narrow at the tip but were occasionally broadened so that in cross-section they had a mushroom-like appearance. In longitudinal section, this appeared as an invagination of the sides of the folds. Secondary or smaller folds occurred between the primary longitudinal folds.

All of the oesophageal folds were covered with a thick layer of stratified epithelium. The surface cells at the tips of the primary folds were characteristically flattened although this was not necessarily true of all folds. In some instances, the surface cells did not differ materially from those of the layers directly beneath. Cells of the deeper layers were often considerably elongated at right angles to the surface. Mucous cells were very numerous in the oesophagus as in the pharynx and presented, for the most part, the same appearance (Fig. 2). They extended only part way up the sides of the primary folds but entirely covered the smaller secondary folds. Nearer the intestine, they became fewer in number, occurring in the deep grooves between the folds and on the sides (Fig. 3). Where they were not closely packed, they were of the typical goblet-cell shape. The contents of the cells had a reticular appearance and gave the specific red color with mucicarmine.

The stratified epithelium of the oesophagus continued almost to the peak of the intestinal sphincter but ultimately gave way to a simple columnar type (Fig. 3). Here, the surface cells gradually disappeared and the basal layer continued over the sphincter as high columnar epithelium with darkly staining cytoplasm.

The tunica propria of the pharynx continued through the oesophagus with little change in appearance. It was difficult to make out a stratum compactum in the oesophagus but it was observed occasionally, especially when the section was stained with Mallory's stain. Where it could be distinguished, it appeared as a wavy line of one or two fibers directly below the basement membrane. The line of demarcation between tunica propria and tunica muscularis was difficult to determine because of invading muscle fibers. These longitudinal striated fibers were not arranged in layers and there appeared to be no fibers in that part of the tunica propria which extended over the sphincter.

The tunica muscularis itself consisted primarily of a thick layer of striated circular muscle with a few longitudinal fibers external to it. The fibers were bound into bundles by connective tissue continuous with that of the tunica propria and serosa. Although this muscle layer was narrower than that of the pharynx, it was much wider than that of the intestine.

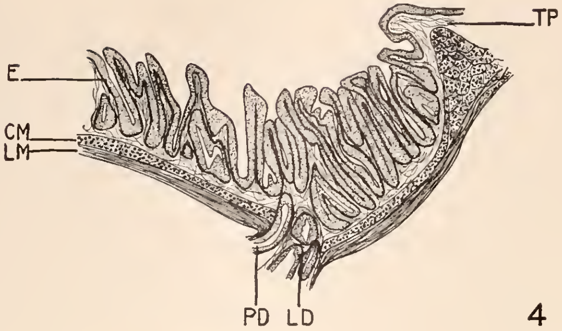
At the junction of oesophagus and intestine, the circular muscle widened suddenly to approximately twice its original width and as abruptly narrowed again (Fig. 3). The muscle fibers were very large, particularly at the periphery of the layer, and the entire band of circular muscle appeared to end at the termination of the sphincter. Small bands or isolated fibers of longitudinal muscle interrupted the continuity of the circular fibers at this point.

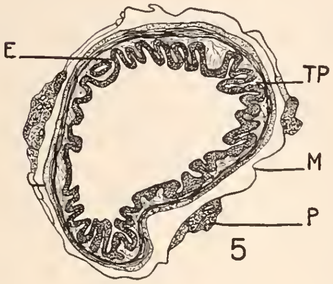
The dorsal diverticulum arose near the beginning of the oesophagus. It extended caudally and tapered to its junction with the pneumatic duct. The proximal portion of the diverticulum was lined with stratified epithelium continuous with that of the oesophagus (Fig. 2). The main part of the cavity was lined with simple columnar epithelium which did not appear to be of the secretory type. Cytoplasm of the cells was granular and the lightly staining nuclei were located toward the bases of the cells. The mucosa formed numerous small folds which lined branching evaginations of the main cavity. A few very short striated muscle fibers were present in the tunica propria. Two layers of muscle of equal thickness, an inner circular and outer longitudinal, comprised the muscular coat. These layers were relatively thin, consisted entirely of striated muscle and were directly continuous with the tunica muscularis of the oesophagus. The tunica serosa also continued from the oesophagus around the diverticulum.

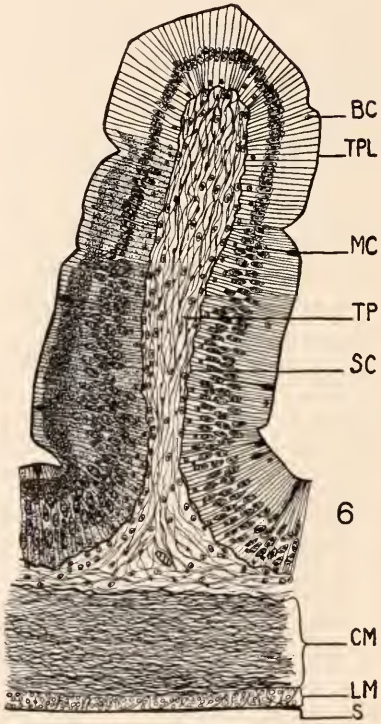
Intestine

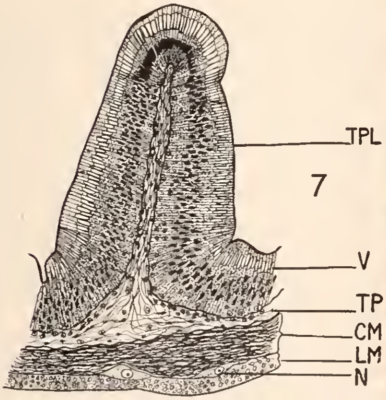
The mucosal folds in the entire intestine, viewed from the surface, followed a herringbone pattern. When the intestine was distended with food, these folds could be clearly seen through the walls. Sections showed that the folds of the intestinal bulb were higher and more complex than those of the intestine proper, often joining with each other above smaller folds (Fig. 4). The deeper portions thus appeared like glands and the folds, like villi. In the main part of the intestine, the folds were shorter and broader and exhibited a much simpler arrangement (Fig. 5). In the anal portion of the rectum, the mucosal folds became again very complicated and branching.

The epithelium of the intestine contained two types of cells, simple columnar and mucous. These cells were covered with a relatively thick top plate and were characteristically very long and narrow. There was, however, some variation in their height; at the apices of the folds they were slightly shorter while along the sides and in the deep portions of the folds, they were very tall. Nuclei of the columnar cells were usually located in the basal third of the cell and were broadly oval. In almost all of the nuclei clumps of chromatin were evident. The cytoplasm stained rather lightly, as a rule, although the cells covering the tips of the folds were considerably darker than the rest. The cytoplasm of all









(See p. 58 for abbreviations)

FIG. 4. Diagrammatic longitudinal section through the first part of the intestinal bulb showing entrance of ducts of the liver and pancreas. This shows the characteristic folding of the mucosa. The sphincter is at the extreme right. × 15.

FIG. 5. Transverse section through the intestine proper showing type of mucosal folds. × 15.

FIG. 6. Transverse section through a fold of the intestinal bulb showing, on one side of the fold, the details of structure of the epithelium. Note that the aggregation of cytoplasmic granules gives a banded appearance. Goblet cells are shown in solid black. × 175.

FIG. 7. Transverse section through a fold of intestine showing vacuole in each cell. × 150.

the cells was slightly granular. In most cases, in the first third or half of the intestine, the granulation appeared to be heaviest in the outer part of the cell and produced a banded effect in the epithelium. There was thus a distinct differentiation between the perinuclear cytoplasm and that in the outer part of the cell (Fig. 6).

In those fish which were killed immediately after feeding, there occurred a definite change in the epithelium in the last half of the intestine. Each cell contained a single vacuole immediately distal to the nucleus (Fig. 7). Cytoplasm was present between the vacuole and the luminal edge of the cell and the vacuoles, therefore, occupied a position in the cells corresponding to the location of heavy cytoplasmic granulation as it occurred in cells of the intestinal bulb and the first part of the intestine proper. In contrast to mucus, which occupied the entire distal end of a goblet cell and caused a distinct bulge in the wall, the vacuoles remained small and occupied a definitely limited area within each cell. They did not stain with any of the stains used but remained perfectly clear. The fact that they showed no reaction to mucicarmine indicated positively that the contents were not mucus. In fact, sections stained with Delafield's haematoxylin showed goblet cells filled with blue-staining mucus at the same time that vacuoles in the columnar cells remained unstained.

Mucous cells were very few in the intestine. The tips of the mucosal folds were generally entirely free of them; the greater number occurred in the deeper parts of the folds. They were about equally numerous in the intestinal bulb and in the intestine proper. They occurred singly and extended the entire width of the epithelium as typical goblet cells. The expanded portion of the cell was located usually at the luminal end but might occur anywhere between the top plate and the basement membrane. That part of the cell which was enlarged with mucus was typically spherical and the basal part containing the nucleus was long and filamentous. When the spherical portion was located below the distal end of the cell, the elongated slender part extended to the lumen (Fig. 6). The staining reactions of the goblet cells indicated that they were similar to the mucous cells of the pharynx and oesophagus.

The epithelium of the rectum resembled that of the oesophagus. Mucous cells were fewer and consequently less crowded, and individual cells in this region were the largest of any in the whole tract.

The tunica propria of the intestine was continuous with that of the oesophagus and formed a much more distinct layer. It was composed of areolar tissue containing fat cells. Mast cells occupied a more conspicuous place in the tissue than in the other parts of the tube, thus substantiating the results of Bolton (1933). A basement membrane was present and, directly beneath it, a few thickened fibers which might be considered the stratum compactum although they did not form a conspicuous layer. Immediately above the muscle, however, was a definite sheet of tissue composed of large, wavy, collagenous fibers. This corresponded more closely in structure to the stratum compactum as figured by Rogick (1931) but its location would define it as submucosa. It arose quite definitely at the intestinal sphincter as a concentration of large longitudinal fibers.

The muscular coat was composed of two layers, an inner circular and an outer longitudinal. They were apparently not continuous with the tunica muscularis of the oesophagus and formed thinner sheets of tissue than in the more anterior parts of the tract (Fig. 5). The longitudinal fibers constituted a definite layer in contrast to the scattered fibers found more anteriorly. The fibers were striated throughout the greater part of the intestinal bulb and a few striated fibers extended into the intestine proper. Smooth muscle gradually replaced the striated and continued through the rectum. Nerve cells between the two muscle layers indicated the presence of a myenteric plexus. A typical serosa covered the entire intestine.

The bile duct and pancreatic duct entered the intestinal bulb a short distance caudal to the intestinal sphincter (Fig. 4). The epithelium of both ducts appeared stratified in some places. Actually, however, it was of the simple columnar type. The sub-epithelial connective tissue and a few striated muscle fibers were apparently continuous with similar tissues in the wall of the intestinal bulb.

Discussion

The foregoing account furnishes a basis for comparison of the digestive tract of *Carassius* with that of other teleosts. In general structure, as well as in most of its details, it resembles closely the tract of the minnow, *Campostoma* (Rogick, 1931). Absence of a stomach suggests a certain similarity with the digestive tube of *Fundulus* but there are marked individual differences. The tract as a whole is considerably longer in *Carassius,* typically twice the body length, whereas in *Fundulus* the two are about equal. This is apparently the result of differences in length of the intestinal portion in the two species. Probably because of this, the intestinal bulb of *Carassius* is longer than the corresponding portion of the tract in *Fundulus* and not so sharply tapering. Variations in intestinal length in these three species may well be correlated with differences in diet. *Carassius* obviously represents an intermediate condition between the extremely long intestine of *Campostoma* and the short intestine of *Fundulus*.

Diversity in descriptive terminology presents difficulties in an exact comparison of the histological structure with the detailed accounts of the histology of the tract in other fishes. In general, however, there would seem to be a close correspondence among the several species which have been described. In particular, the epithelium of the pharynx and of the true intestine appears similar in *Carassius, Campostoma* (Rogick, 1931) and *Pleuronectes* (Dawes, 1929). Blake does not include a description of the pharynx in his account of *Centropristes* or *Prionotus* (1930, 1936). The oesophageal epithelium of these forms, however, is similar to that of *Carassius* and *Campostoma* and the similarity in structure of the intestine is striking.

The histological study is of particular significance in determining that at no point in the tract are there cells which resemble the cells of gastric epithelium. A special interest, therefore, attaches to the cells of the intestinal mucosa.

Both Rogick (1931) and Dawes (1929) describe in some detail the columnar cells of the intestinal epithelium. Dawes found that the resting cell contained a darkly-staining mass in the center. During active digestion, this mass disappeared but an area of darker cytoplasm appeared below a pale-staining border in each cell. The nuclei, likewise, exhibited differences in staining reaction under the two conditions. In her figure of the intestine, Rogick showed a clear area in approximately the middle of the cytoplasmic portion of each columnar cell. The darkly-staining cytoplasmic band and the clear area have been found in the intestinal epithelium of *Carassius,* the former in the intestinal bulb and upper intestine and the latter, in the lower part of the intestine proper. Rogick considers the clear vacuoles to be an early stage in the accumulation of mucus within the cell but the investigation on *Carassius* throws considerable doubt on this interpretation. In addition to the failure of these vacuoles to give the specific mucous reaction is the fact that no intermediate stages between these cells and typical goblet cells could be found. Moreover, no such vacuoles were found in cells of the pharynx and oesophagus where the mucous type was the most abundant. With one exception, vacuoles did not appear in the epithelium of fish which had been starved. This, coupled with the findings of Dawes, would indicate a possible relationship between the appearance of vacuoles and the presence of food in the alimentary tract. Unfortunately, no histological study accompanied the work of Babkin and Bowie (1928) on the physiology of digestion in *Fundulus.* Such a combined study would undoubtedly be of considerable value in determining the function of these cells.

Summary

1. The digestive tube of *Carassius auratus* closely resembles that of *Campostoma anomalum* (Rafinesque).

2. Study of the gross structure brought out the following characteristics: the posterior pharynx possesses pharyngeal teeth which bite on a dorsal horny pad; a dorsal diverticulum leading to the pneumatic duct extends from the short oesophagus; the oesophagus opens directly into an expanded part of the intestine, the intestinal bulb; a circular sphincter marks the boundary between oesophagus and intestinal bulb; the intestine proper does not show the extreme coiling of *Campostoma* but is considerably longer than that of *Fundulus;* it leads into the rectum.

3. The walls of the entire tract possess a tunica mucosa, tunica muscularis and tunica serosa. Absence of a muscularis mucosae prevents the determination of a distinct submucosa.

4. Epithelium of the pharynx is stratified with numerous mucous cells in the superficial layer. At the intestinal sphincter, there is an abrupt change to the simple columnar epithelium of the intestine. Stratified epithelium with mucous cells appears again in the rectum.

5. Columnar cells of the intestinal bulb and upper intestine show a band of darkly-staining cytoplasm between the nucleus and the luminal edge of the cell. In the lower half of the intestine, this region of each columnar cell is marked by a single vacuole which does not react to any of the stains used.

6. The tunica propria of the posterior pharynx and oesophagus is invaded with muscle fibers from the muscular layer. It forms a distinct region in the intestine. A stratum compactum, while distinguishable, is not a conspicuous feature. Mast cells are present, particularly in the intestine.

7. Striated muscle forms the tunica muscularis of posterior pharynx, oesophagus and intestinal bulb. It is gradually replaced by smooth muscle in the intestine proper. Between the oesophagus and intestinal bulb, the circular layer of muscle becomes enlarged to form the intestinal sphincter.

8. A serosa covers the entire tract beyond the most posterior part of the pharynx.

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STUDIES ON A CORTICAL LAYER RESPONSE TO STIMULATING AGENTS IN THE ARBACIA EGG

III. Response to Non-Electrolytes

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Introduction

According to Moore (1930*a*, 1930*b*), and Moore and Moore (1931), eggs of *Strongylocentrotus purpuratus, Dendraster eccentricus,* and *Paracentrotus lividus*, treated with non-electrolyte solutions isosmotic with sea water, are rendered incapable of " forming " fertilization membranes upon subsequent insemination in sea water. Moore suggested that this loss of ability to elevate a membrane was " the result of the diffusion of something from the egg which renders membrane formation impossible." Moreover, the " power of forming membranes once lost cannot be regenerated by the egg." In his experiments Moore generally used molar urea solution, pH 7, although similar results were obtained with both glycerine and sucrose solutions. Chase (1935) reported evidence in confirmation of Moore's results on *Strongylocentrotus purpuratus,* and *Dendraster eccentricus.*

In the first two papers of the present series (Moser, 1939*a*, 1939*b*) it was shown that stimulation of the *Arbacia* egg results in the breakdown of a thin layer of cortical granules. It was postulated that vacuoles arising from the breakdown of the cortical layer granules play a part both in the elevation of the fertilization membrane as well as in the other related visible cortical changes which occur at the stimulated egg surface. It was suggested further, in terms of the theory of stimulation and response advanced by Heilbrunn and his students (Heilbrunn and Daugherty, 1933; Heilbrunn, 1937), that an initial rapid, and doubtless invisible cortical response led not only to the above-mentioned visible phenomena at the egg surface, but also to the colloidal changes associated with cleavage. According to this concept, membrane elevation and the processes leading to segmentation are related only in that both depend upon a common reaction. It is therefore conceivable that this reaction, which, presumably, is common to both membrane elevation and cleavage might initiate either response without calling forth the other. Essentially in accord with this idea are Glaser (1913), Loeb (1915), Lillie (1919), Gray (1922), Just (1919*a*, 1923), and Carter (1924).

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If, now, it be assumed that the above theory is correct, and in addition that the forms with which Moore has worked represent systems which are comparable to the *Arbacia* egg and also that the loss of membrane "forming" ability is the result of the diffusion of some substance from the egg, then the failure of the membrane to elevate in urea-treated eggs, may be attributed to the loss of a substance which initiates the visible cortical phenomena. But if this be true, then how are we to explain the fact that cleavage takes place when these eggs are inseminated, for according to the theory both the visible cortical response and segmentation are initiated by the same substance? With this problem in mind, experiments similar in some respects to those of Moore have been performed.

Acknowledgments

I wish to take this opportunity of expressing my gratitude to Dr. L. V. Heilbrunn, of the University of Pennsylvania, for his kind interest and helpful criticisms rendered during the progress of this work.

Material and Methods

Arbacia eggs and dry sperm were secured in the usual manner.

A molar urea solution, pH 6.8 (indicator method), was used in most of the experiments. In a few cases, molar solutions of thiourea, glycerine or sucrose were used.

The procedure of exposing *Arbacia* eggs to non-electrolyte solutions was somewhat similar to that of Moore (1930*a*). One or 2 cc. of a thick suspension of eggs was pipetted into 50 cc. of the non-electrolyte solution. The eggs were exposed to the action of this solution for 3–5 seconds, 30 seconds, 1, 1½, 2 and 2½ minutes. Whenever the treatment with non-electrolyte lasted for more than one minute, the eggs were transferred to a second 50 cc. of the same non-electrolyte at the end of the first minute. After exposure to the non-electrolyte solution, the eggs were transferred to sea water and divided into two lots, one of which was inseminated. It is perhaps needless to say that the necessary precautions were followed in order not to contaminate the uninseminated lot with sperm cells.

In order to study the effect of an agent upon the egg cortex in terms of the breakdown of the cortical layer granules, it is necessary to shift the underlying endoplasmic granules to one side of the egg. This was accomplished by means of an electric centrifuge which developed a force of approximately 6,000 times gravity. When the eggs are centrifuged until they show five distinct strata, the granular cortex becomes perfectly visible over the clear hyaline zone. The effects of non-electrolyte solutions on the egg cortex were observed by placing a drop of the centrifuged eggs and several drops of the non-electrolyte solution side by side on a glass slide which had previously been fixed in position on the stage of the microscope. The non-electrolyte solution was then made to flow into the drop of eggs and the visible effects noted.

During the period when these experiments were made, room temperature ranged from 21.0° to 27.0° C.; for any one set of experiments the temperature remained fairly constant.

Observations and Results

Cleavage and Membranes of Urea-treated Eggs

The results obtained in one set of experiments, after treatment of *Arbacia* eggs with molar urea solution for various time intervals, are summarized in Table I. Upon examining Table I it becomes apparent that molar urea solution acts as a parthenogenetic agent. Since cleavage occurs at a relatively slow rate after activation with non-electrolyte solutions, the eggs were counted from 4½ to 6½ hours after treatment. The percentages given in the tables are based upon counts of one hundred or more eggs. Cleavage in the uninseminated and the inseminated lots of eggs is quite irregular, except in those cases among the inseminated eggs which have given rise to blastulae. Generally no more than eight or nine blastomeres develop in any one case (blastulae excepted), and usually the number is less.

Microscopic examination of the irregularly cleaving eggs which have been exposed to urea for from ½ to 1½ minutes, reveals that nearly all of these eggs possess thin fertilization membranes. The degree of thinness of the membrane seems to be a function of the length of time that the eggs have been treated with urea. Thus, in those eggs which were treated for ½ minute, the membranes are more easily discerned than those of eggs which had been exposed for 1 minute, and these latter membranes can be seen more easily than those of eggs treated for 1½ minutes. In other experiments in which the eggs had been exposed for from 3–5 seconds, apparently normal membranes were visible when these eggs were examined shortly thereafter in sea water. Indeed, eggs treated for such short time intervals cannot be distinguished from normally inseminated ova. After exposures of two or more minutes usually no vestige of the membrane can be seen.

In addition to the fact that fertilization membranes may be seen in both the inseminated and the uninseminated lots of eggs after treatment with urea for time intervals of from ½ to 1½ minutes, it may be of interest to point out that these membranes are rather closely applied to the egg surface. In some cases such membranes can be identified only in those areas where they stretch between adjacent blastomeres. This is especially true for exposures of 1½ minutes.

The blastulae which develop in the inseminated lot of eggs after treatment for more than 1½ minutes do not appear to have fertilization membranes. The normal relationships of the cells making up the blastulae are, however, retained. Possibly a very thin remnant of the hyaline plasma membrane holds these cells together. In occasional experiments, when the treatment with non-electrolyte solution lasted for two or more minutes, the blastomeres of both the uninseminated and the inseminated lots of eggs become separated; only in one or two exceptional cases did this occur with exposures of 1½ minutes.

TABLE I

Percentage of cleavage and of blastulae in uninseminated and inseminated lots of eggs after treatment with molar urea solution for various time intervals. The number of blastulae is given in terms of the percentage of eggs cleaving rather than in terms of the total number (cleaving plus non-cleaving) of eggs. (Room temperature 23.0° C.)

Time of exposure to molar urea solution	Uninseminated lot		Inseminated lot	
	Percentage cleavage	Percentage blastulae of those cleaving	Percentage cleavage	Percentage blastulae of those cleaving
minutes	*per cent*	*per cent*	*per cent*	*per cent*
0.5	83	0	90	5
1.0	93	0	96	10
1.5	96	0	96	36
2.0	95	0	96	33
2.5	94	0	93	28

Fertilized untreated controls 94 per cent blastulae, 5 per cent in early cleavage, all with membranes. Unfertilized untreated controls 100 per cent without membranes and non-cleaving.

Fertilization Superposed upon Incomplete Artificial Activation

The percentage of cleavage in the uninseminated and the inseminated lots of eggs (Table I) is very nearly the same. Now, since the percentage of blastulae among the uninseminated lot of eggs is zero, while the percentage of blastulae in the inseminated lot is quite high, it would seem that fertilization has been superposed upon incomplete artificial activation. It is evident, moreover, that after exposure to urea of ½ to 1 minute, the percentage of blastulae is considerably lower than that which occurs after exposures of from 1½ to 2½ minutes. One might have expected that just the reverse would be true; that with the shorter treatment with urea, the percentage of superposed fertilization would be greater. It will be remembered, however, that the fertilization membrane is most easily seen after the shorter intervals of exposure to the non-electrolyte solution. It would seem possible, therefore, that the relatively low percentage of blastulae found among the cleaving eggs of the inseminated lot, after treatment for ½ to 1 minute, is due to the fact that the membrane may act as a block to the entrance of sperm cells in these instances, while with the longer exposures this block is partially or completely removed.

However, it must not be assumed, as Table I might lead one to believe, that the percentage of cleavage in the uninseminated and inseminated lots of eggs always approximate each other. If among the uninseminated eggs it is evident that a certain percentage of the eggs is dividing, then it may be expected that the same or a higher percentage of eggs will be dividing in the inseminated lot. Thus in Table II the value 55 per cent cleavage, in the uninseminated lot, after treatment with urea for 3–5 seconds, approximates 54 per cent cleavage in the inseminated lot. Likewise, after exposure to urea of 0.5 minute, the value 85 per cent cleavage, in the uninseminated lot of eggs, is very near to that of 86 per cent in the inseminated lot. However, the next two values, 97 per cent and 95 per cent, in the inseminated lot of eggs, are significantly higher than the corresponding percentages in the uninseminated lot. This increase in the percentage of cleavage in the inseminated eggs as compared to the uninseminated eggs is probably due to the activation of eggs which with the urea treatment alone would not have divided.

Fertilization (Urea-activated) Membrane as Block to Insemination

A trend which was indicated in the column of figures under the heading " Percentage blastulae of those cleaving " in the inseminated lot of Table I is more definite in the same column of Table II. That is, as the time of exposure to the non-electrolyte solution increases, there is a definite tendency for the percentage of blastulae among the cleaving eggs of the inseminated eggs to increase. This, as earlier pointed out, is doubtless due to the presence of the fertilization membrane, which becomes less and less of a block to the entrance of spermatozoa as the exposure time to the non-electrolyte increases. For example, the figure zero under the heading " Percentage blastulae of those cleaving " in the inseminated lot of eggs of Table II is perhaps especially significant, for it will be remembered that exposures of 3–5 seconds produce eggs whose membranes very closely simulate those of normally fertilized ova. Moreover, the increasing percentages, 69 per cent, 87 per cent, and 91 per cent after exposures of 0.5, 1.0, and 2.0 minutes respectively, are indicative, since the membranes become thinner and finally disappear as the exposure time increases.

In exceptional batches of eggs the urea had no effect other than to initiate the reaction leading to the elevation of the membrane. Thus in such an instance, exposures of 3–5 seconds, 0.5 minute, 1.0 minute and 2.0 minutes yielded no cleavage in the uninseminated lot of eggs, except after the 0.5 minute interval; segmentation occurred in only 1.8 per cent of these latter eggs. In the inseminated lot, however, as many as 93 per cent of the eggs gave rise to blastulae; clearly as a consequence of the added stimulus of fertilization.

TABLE II

Percentage of cleavage and of blastulae in uninseminated and inseminated lots of eggs after treatment with molar urea solution for various time intervals. The number of blastulae is given in terms of the percentage of eggs cleaving rather than in terms of the total number (cleaving plus non-cleaving) of eggs. (Room temperature 25.0° C.)

Time of exposure to molar urea solution	Uninseminated lot		Inseminated lot	
	Percentage cleavage	Percentage blastulae of those cleaving	Percentage cleavage	Percentage blastulae of those cleaving
	per cent	*per cent*	*per cent*	*per cent*
3–5 sec.	55	0	54	0
0.5 min.	85	0	86	69
1.0 min.	85	0	97	87
2.0 min.	81	0	95	91

Fertilized untreated controls 100 per cent blastulae. Unfertilized untreated controls 100 per cent without membranes and non-dividing.

Direct Observations on Response of Egg Cortex to Urea

From the observations already presented in this paper, it is evident that the shorter exposures of *Arbacia* eggs to molar urea solutions result in the elevation of a fertilization membrane, which is readily visible when the eggs have been placed in sea water. After the longer exposures, it is impossible to see the fertilization membrane. Relative to these facts, a number of possibilities suggest themselves. In the first place the short exposures might initiate a change at the egg surface which would result in the breakdown of the cortical layer granules (see Moser, 1939*a*, 1939*b*) only after the eggs have been placed in sea water. According to this concept, the longer exposures would in some way inhibit the cortical response when the eggs are subsequently returned to sea water. As a second possibility, the cortical layer granules might break down and membrane elevation occur during the period of exposure to the non-electrolyte. If the latter possibility be true, then after the longer exposures, it is impossible to see the fertilization membrane, not because membrane elevation has been inhibited, but because it has disappeared subsequent to the process of elevation.

Earlier observations have suggested that the latter of the above possibilities is the correct one. The results which follow furnish absolute proof in this direction. Thus, when four to five drops of a molar urea solution were made to flow into a drop of centrifuged or uncentrifuged eggs, the cortical layer granule breakdown response was observed. This response to urea is essentially the same as that obtained in the presence of sperm cells (Moser, 1939*a*) and other stimulating agents (Moser, 1939*b*; *see* also Moser, 1937, for preliminary report on the effect of urea). The initial visible cortical changes are wave-like in both the centrifuged and uncentrifuged eggs. The direction of the wave apparently depends upon the direction of flow of the non-electrolyte solution. For example, when the urea flowed into the drop of eggs from the right side of the microscopical field, then the visible cortical response progressed from right to left over the egg surface as viewed under the microscope.

Generally, immediately following the breakdown of the cortical layer granules, rapid elevation of the fertilization membrane occurs. In many cases the perivitelline space is from two to three times as wide as in normal fertilization. If at this stage, or immediately after the initial visible cortical response the eggs are placed in sea water, then the membranes appear to be almost normal, although they may be somewhat thinner and further removed from the egg surface. In the event that the eggs are not removed to sea water immediately after membrane elevation, the membranes begin to recede toward the egg surface and gradually become thinner, until finally in most cases no vestige of the membrane can be seen. If these eggs are then placed in sea water, many of them exhibit the early cleavage stages. The cleavages are very irregular and in most cases do not exceed eight or nine cells. Sometimes the blastomeres are held together by means of a thin film which is probably the hyaline plasma membrane, while in other instances the blastomeres are strung out somewhat in the manner of a colony of yeast cells.

Most of the above-described observations have been made on eggs treated with molar urea solutions. Similar results have been obtained in response to molar solutions of thiourea, glycerine, and sucrose.

Amoeboid Activity of Urea-treated Eggs

It may be of some interest to point out that eggs which are not removed from the urea solutions do not cleave, though they do exhibit a form of protoplasmic activity which may be but a variation of the normal cleavage process. Thus, shortly after *Arbacia* eggs have been placed in urea solution, they not only lose their spherical shape, but indeed exhibit a peculiar form of amoeboid movement. Characteristically a rather large portion of the surface of the egg suddenly rushes outward followed immediately by endoplasmic material which flows into and with the outward moving surface layer. Generally no two such bursts of movement succeed each other at the same portion of the egg. A second burst of movement may occur at a point immediately adjacent to the previous one, or anywhere between the preceding locus of movement and the opposite pole of the cell. For the most part this rather random activity of amoeboid *Arbacia* eggs results in rotatory movement alone, though in some instances translatory movement over short distances does occur.

After the eggs have been in a molar urea solution for approximately two hours, clear blebs and vesicles begin to form at their surfaces. These vesicles, which in many instances become separated from the eggs, vary from a few microns in diameter to a size which approaches that of the egg diameter itself. Concurrently with the formation of the clear vesicles, the amoeboid activity of the eggs becomes decidedly slower, and during the next hour movement ceases entirely.

DISCUSSION

The observations presented in this paper make it possible to select some data for which both the experimental procedure and the results parallel those of Moore (1930*a*, 1930*b*), and Moore and Moore (1931). Thus when eggs are treated with molar urea solution for two or more minutes and are then transferred to sea water in which they are subsequently inseminated, cleavage is found to take place as well as the development of rather normal looking blastulae. Moreover, these dividing cells do not possess a fertilization membrane, though they may be held together by a thin remnant of the hyaline plasma membrane. From such data alone, one might readily conclude that treatment with molar urea solution results in the inhibition of fertilization membrane elevation even though the processes of fertilization and cleavage still occur.

Such a conclusion becomes untenable, however, in the light of other observations and results, for it has been shown that non-electrolyte solutions initiate not only the visible phenomena at the surface of the egg but the process of cleavage as well. The fact that one cannot see the fertilization membrane upon insemination after a two-minute treatment with urea is not a consequence of the inhibition of membrane elevation, but rather it is due to the fact that membrane elevation has been initiated and the membrane then dissolved by the very substance responsible for the reaction leading to its elevation.

It is possible that the forms with which Moore (1930) has worked represent systems which differ from the Arbacia egg. If, however, it be true that these forms are similar, then the fact that membrane elevation was not obtained upon insemination after treatment with urea in the case of *Strongylocentrotus purpuratus, Dendraster eccentricus,* and *Paracentrotus lividus,* might be explained by assuming that here, as in the *Arbacia* egg, membrane elevation and the dissolution of the membrane had already taken place in the urea solution. Thus the question of how non-electrolyte solutions prevent membrane elevation either irreversibly or otherwise completely loses significance. Moreover, the blastulae, however plate-like, obtained in the above-mentioned forms, may have been the result of fertilization superposed upon incomplete artificial parthenogenesis. In agreement with the concept of the superposition of fertilization upon incomplete artificial activation are the papers of C. R. Moore (1916, 1917), Just (1919*b*, 1922), and Lillie (1921). However, these workers have presented evidence which indicates that membrane elevation in itself is the result of a complete cortical response.

Again with regard to the elevation of the fertilization membrane, Motomura (1934, 1938) has shown that eggs of *Strongylocentrotus nudus, Strongylocentrotus pulcherrimus* and *Strongylocentrotus depressus* elevate membranes when placed in sea water or simple salt solutions after previous treatment with molar urea for from four to ten seconds. Thus, he (Motomura, 1938) says, " When eggs of a sea urchin are previously activated with urea solution, they form the fertilization membrane parthenogenetically even in simple salt solutions of monovalent and divalent cations." According to this concept, the membrane does not elevate in the urea solution itself, but is conditioned by a second treatment with sea water or " simple salt solution." With these forms, as with the Arbacia egg, it is likely that membrane elevation occurs in the urea solution and that the dissolution of the membrane is prevented when the eggs are returned to sea water or the " simple salt solutions." This seems especially possible in view of Motomura's (1934) conclusion, "The loss of capacity of membrane formation can be caused by treating the eggs for a long time either with isotonic urea solution or with butyric acid sea water."

Amoeboid movements of egg cells have previously been described. Thus, Lillie (1902) and E. B. Harvey (1939) have shown that the eggs of *Chaetopterus pergamentaceus* exhibit such activity under certain experimental conditions. Churney (1940) describes amoeboid movements of fertilized *Arbacia* eggs treated with "pure solutions of potassium magnesium." While an explanation of the amoeboid activity of *Arbacia* eggs treated with urea will not be attempted, yet the phenomenon is of some special interest since it occurs in cells whose normal function is to divide. Indeed these movements may be an abortive form of cleavage furrow formation, an idea which falls in line with the concept, advanced by Marsland (1936, 1938, 1939) and Schechtman (1937), that cleavage furrow formation and amoeboid movement are but two expressions of the same general type of protoplasmic activity.

Summary

1. *Arbacia punctulata* eggs treated with molar concentrations of non-electrolyte solutions (urea, thiourea, glycerine or sucrose) exhibit essentially the same kind of visible cortical response as that obtained with sperm cells and other stimulating agents.

2. Typically the cortical response to non-electrolyte solutions results in the formation of a perivitelline space which is from two to three times as wide as that obtained upon insemination.

3. Continued exposure to non-electrolyte solution following the visible cortical response results in the dissolution of the fertilization membrane.

4. Since activation, however incomplete, is obtained upon treatment with non-electrolyte solutions, these solutions may be regarded as being artificial parthenogenetic agents.

5. Fertilization has been superposed upon the incomplete activation obtained in the presence of non-electrolyte solutions.

6. Within certain limits, as the length of exposure to the non-electrolyte solution increases, an increase in the percentage of blastulae among the inseminated treated eggs occurs; this fact may be correlated with (3) above.

7. It has been suggested that results reported by Moore (1930*a*, 1930*b*), Moore and Moore (1931), Motomura (1934, 1938), and Chase (1935) may be explained on the basis of the observations and results presented in this paper.

8. The amoeboid activity of *Arbacia* eggs treated with urea has been briefly described.

9. The observations and results presented in this paper are essentially in agreement with the views considered in the earlier papers of the present series (Moser, 1939*a*, 1939*b*).

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STUDIES ON A CORTICAL LAYER RESPONSE TO STIMULATING AGENTS IN THE ARBACIA EGG

IV. Response to Chemical and Physical Agents in the Absence of Oxygen, and Observations of the Effects of Low Oxygen Tensions and High Hydrostatic Pressures upon Amoeboid Eggs

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Introduction

In response to insemination, or to treatment with certain chemical and physical agents, the cortical layer of the *Arbacia* egg undergoes characteristic irreversible changes (Moser, 1939*a,* 1939*b*). A thin layer of cortical granules breaks down and elevation of the fertilization membrane follows immediately. An interpretation of this and of other related phenomena has been suggested (Moser, 1939*a,* 1939*b*) in terms of the more general theory of stimulation which Heilbrunn and his students have developed (Heilbrunn and Daugherty, 1933; Heilbrunn, 1937).

The experiments described in this paper have been in part devoted to an investigation of the cortical response in the absence of oxygen (see also Moser and Kitching, 1939). *Arbacia* sperm is immobilized in the absence of oxygen, so that fertilization and the resulting membrane elevation do not occur (E. B. Harvey, 1930; Barron, 1932). However, it seemed possible that the cortical response might take place without oxygen if the eggs were subjected to an adequate stimulus. We have therefore investigated, in the absence of oxygen, the effects of certain chemical and physical agents which in air are known (Moser, 1937, 1939*b*) to produce the cortical response.

It is already well established (Loeb, 1895; E. B. Harvey, 1927; Amberson, 1928) that cleavage cannot take place without oxygen or at very low oxygen tensions. However, in view of the definite relationship which seems to exist between the initial cortical response and the subsequent cleavage of the egg (see, for example, Loeb, 1915*a;* R. S. Lillie,

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1915; F. R. Lillie, 1919; Chambers, 1921*a*, 1921*b;* Just, 1919, 1923; and Moser, 1939*b*), we have tried to determine whether the cortical response initiated in the complete absence of oxygen could lead to cleavage when the eggs have been returned to air.

Unfertilized *Arbacia* eggs placed in isotonic urea solution become aspherical and angular in outline within a few minutes and later undergo a peculiar form of amoeboid movement (Moser, 1940; see also Churney, 1940 for amoeboid movements of fertilized *Arbacia* eggs). These movements are mostly rotatory, although sometimes short distances are traversed. *Amoeba proteus* (Hulpieu, 1930) and marine limacine amoebae (Pantin, 1930) slow down and finally stop in the absence of oxygen; and there is recovery in air. The stoppage of movement is almost immediate in the marine amoeba *Flabellula mira* (Kitching, 1939*b*). The amoeboid movement of *Arbacia* eggs is an abnormal activity of a cell whose normal function is cleavage. It seemed, therefore, to be of interest to compare the effects of low oxygen tensions on the normal cleavage and on the amoeboid movement of *Arbacia* eggs.

In addition high hydrostatic pressure inhibits furrow formation of *Arbacia* eggs (Marsland, 1938, 1939) and amoeboid movement of amoebae (Marsland and Brown, 1936). The action of high hydrostatic pressure is attributed to a liquefaction of gelated cortical protoplasm. We have investigated the effect of this agent on the amoeboid movement of unfertilized *Arbacia* eggs treated with urea. We are grateful to Mr. Daniel C. Pease, of Princeton University, for placing his pressure apparatus at our disposal.

Acknowledgments

This work was carried out at the Marine Biological Laboratory, Woods Hole, for the facilities of which we are grateful. We also wish to thank for their interest and criticism Professor E. N. Harvey, of Princeton University, and Professor L. V. Heilbrunn, of the University of Pennsylvania.

Methods

The general procedure was to subject *Arbacia* eggs under anoxic conditions to treatment which in air is known to elevate fertilization membranes. In addition some observations at various known oxygen tensions were made on the amoeboid movements of eggs in urea, and on cleavage. Eggs therefore had to be kept under microscopical observation while exposed to suitable gas mixtures; and transfer from one solution to another had to be accomplished without leakage of gas to or from the exterior.

Arbacia eggs were obtained in the usual manner. The percentage of membrane elevation after normal insemination was determined for each batch of eggs. Only those batches which yielded 97 per cent to 100 per cent membranes were used in these experiments.

A special glass observation chamber was used (Fig. 1). Besides the usual inlet and outlet for gas, there was a wide bent side-arm which allowed free movement of an iron wire actuated by a Chambers' micromanipulator, but which when filled with mercury did not allow any interchange of gas between the chamber and the outside. To the end of the wire which projected into the chamber was attached with de Khotinsky cement either a micro-needle or a fine glass loop. A cover slip was sealed with vaseline over the chamber, and eggs could be transferred by means of the loop from one hanging drop to another. In this way eggs could be subjected to a fairly complicated treatment. When several hanging drops were required the cover slip was usually coated with a monolayer of ferric stearate to prevent coalescence, as suggested to us by Mr. R. Ballentine. For this purpose one drop of ferric stearate in benzene was evaporated on the cover slip; and the cover slip was then heated sufficiently to melt the ferric stearate, and finally wiped as clean as possible. The gas leads to and from the chamber were of reasonably flexible lead tubing, so that the chamber could be actuated by the mechanical stage of the microscope, and could also be shaken sufficiently vigorously to make the hanging drops flow together.

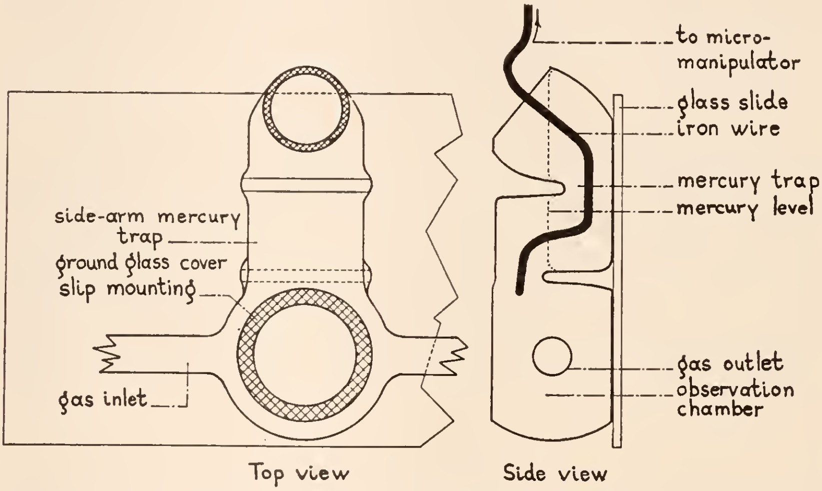
to micro-manipulator
glass slide
iron wire
mercury trap
mercury level
side-arm mercury trap
ground glass cover slip mounting
gas inlet
gas outlet
observation chamber
Top view
Side view

FIG. 1. Drawing of special glass observation chamber (ca. × 1).

The chamber was flushed either with hydrogen purified over platinized asbestos, or with known mixtures of purified hydrogen and oxygen. The apparatus for this purpose is described elsewhere (Kitching, 1939*a*). The gas flowing out from the observation chamber was carried by lead tubing to a water trap.

Another chamber for control experiments was flushed with carbon dioxide-free air. In this way the possible influence of alkalinity of the sea water, due to loss of carbon dioxide, was taken into account.

A hydrostatic pressure apparatus with modifications introduced by Pease and Kitching (1939) was used in the experiments with amoeboid *Arbacia* eggs. Concurrent observations were made on eggs at atmospheric pressure and on eggs in the pressure chamber at high pressure.

RESULTS

Membrane Elevation in the Absence of Oxygen

Saponin.—In each of four experiments a thin hanging drop of sea water containing unfertilized *Arbacia* eggs was placed close to a drop of 0.25 per cent saponin in sea water. The cover slip was mounted over the observation chamber, and pure hydrogen was flushed through continuously at 50–75 cc. per minute. After an interval ranging from 25 to 100 minutes the chamber was shaken (with the hydrogen still running) until the drops flowed together. Membrane elevation occurred within two or three minutes, just as in control eggs similarly treated in carbon dioxide-free air. The times taken for the surface reaction to occur, as shown by a roughening of the surface (see Moser, 1939*b*), for the beginning of elevation, and for the satisfactory elevation of a good membrane, were alike in experimental and control eggs. In both cases cytolysis occurred after some minutes.

Urea.—The effects of isotonic urea upon unfertilized *Arbacia* eggs in air have been described by Moser (1937, 1940). In two experiments a hanging drop of unfertilized eggs in sea water was surrounded by several drops or by a ring of 1.0 molar urea. In this way the ratio of urea to sea water was made large without the disadvantages of a thick drop. After equilibration with pure hydrogen (for seventy minutes in one experiment and eighty in the other) the drops were mixed. Membrane elevation started within fifteen seconds, and within two or three minutes the membranes shrank and disappeared. The eggs were, however, distinguishable by their minutely rough surface from normal unfertilized eggs. Similar results were obtained with eggs treated in air and carbon dioxide-free air. These results are confirmed by later experiments described below.

Sucrose.—In one experiment with sucrose, set up and carried out just as in the case of the experiments with urea, the surface reaction took place without oxygen within twenty seconds, but no membranes were visible. Some of the eggs showed characteristic amoeboid movement (see below) when transferred to carbon dioxide-free air.

Hypertonic Sea Water.—In air, treatment with hypertonic sea water for suitable times, followed by return to ordinary sea water, leads to the appearance of a fertilization membrane (Loeb, 1913). E. B. Harvey (1936) has used 30 grams of sodium chloride in one liter of sea water, and we have followed her formula. In two experiments, after suitable equilibration with hydrogen, unfertilized eggs were transferred with the loop first to hypertonic sea water for about twenty minutes and then to ordinary sea water. A peculiar alveolar structure developed in the cortex. This phenomenon has already been described by Hunter (1936). Similar treatment in carbon dioxide-free air led to membrane elevation in some eggs and to cytolysis in others. Eggs treated in the absence of oxygen first with urea, then with ordinary sea water and finally with hypertonic sea water, did not develop the alveolar cortex, although a number cytolyzed.

Puncture.—When a centrifuged unfertilized *Arbacia* egg is pricked with a micro-needle at any point over the centripetal pole, a characteristic wave of cortical granule breakdown, accompanied by membrane elevation, spreads over the surface (Moser, 1939*b*). Cytolysis rapidly ensues. A number of eggs punctured in hydrogen, and others in carbon dioxide-free air, gave the typical response.

Cleavage and Amoeboid Eggs

Cleavage in Air after Anoxic Chemical Treatment.—If unfertilized *Arbacia* eggs are transferred first to urea for a few minutes, and then back to sea water, the cortical reaction is followed in three or four hours by an irregular cleavage (Moser, 1937, 1940). In these experiments unfertilized eggs after equilibration for an hour in pure hydrogen were transferred to urea for three minutes, and then to three successive washes of sea water; each of the washings lasted for ten minutes. After the eggs had been in the third drop of sea water for ten minutes, the cover slip was placed over an observation chamber in air. Cleavage then occurred in from two to five hours. Although cleavage was irregular, there was no doubt as to its genuine nature, for the nuclei of the daughter cells were clearly visible. The eggs which had remained in the drop of urea solution (having fallen out of the loop) became irregular and finally underwent characteristic amoeboid movement (see below). In another experiment the eggs were treated in the same manner as those described above, but were not transferred to air; no cleavage or amoeboid movement occurred in these eggs.

The Effect of Low Oxygen Tensions on Cleavage and Amoeboid Movement.—A series of experiments were carried out in which unfertilized eggs in urea and fertilized eggs in sea water were set side by side in two hanging drops on the same cover slip. The drops were set up as soon as possible after treatment with urea or insemination, and exposed over the observation chamber to known mixtures of hydrogen and oxygen. Control experiments were carried simultaneously in carbon dioxide-free air and in ordinary air. The results are shown in Table I. At 4.4 mm. of oxygen cleavage was delayed as compared with control eggs, while at 0.95 mm. the eggs entirely failed to cleave. We have not attempted to determine accurately the minimal tension at which cleavage can occur, but this has already been given by Amberson (1928) as 4 mm. Active amoeboid movement took place at oxygen tensions too low for cleavage, and some slight movement was even seen at 0.42 mm. oxygen, while at 0.95 mm. amoeboid movement was active. Fertilized eggs became somewhat wrinkled and shrunken in appearance (as

TABLE I

The effects of various oxygen tensions upon cleavage in sea water and upon amoeboid *Arbacia* eggs in urea solution. (Room temperature 21.9–24.4° C.)

Experimental Eggs			Control Eggs (in air)	
Oxygen tension in mm. Hg	Cleavage in sea water	Amoeboid movement in urea solution	Cleavage in sea water	Amoeboid movement in urea solution
12.6	Normal	†Active	Normal	Active
6.4	Normal	Active	Normal	Active
4.4	Delayed	Active	Normal	Active
0.95	Eggs wrinkled; no cleavage; finally cytolysis.	Active	Normal	Active
0.42	Eggs wrinkled; no cleavage; finally cytolysis.	A few eggs exhibit very slow amoeboid movement.	Normal	Active
*0.00	Eggs wrinkled; no cleavage.	—	Normal	—

* Pure nitrogen and not hydrogen was used in this experiment.

† The term "active" indicates that all or nearly all of the eggs exhibit the rather sudden changes in shape which have been characterized by Moser (1940, pp. 75–77).

described by E. B. Harvey, 1927) at oxygen tensions too low for cleavage, or in pure hydrogen. In one experiment a similar result was obtained in pure nitrogen.

Amoeboid eggs in urea solution, as already shown by Moser (1940), finally cease movement; and clear vesicles or "blebs" of various sizes develop and protrude from the surface. This happened in our experiments also, both in air and in mixtures of oxygen and hydrogen. However, in pure hydrogen (in which the eggs remained spherical) the blebs did not develop. Instead a clear layer of uniform thickness formed between the surface membrane and the granular cytoplasm. This layer presumably corresponds to the clear vesicles which develop on amoeboid eggs, and in fact somewhat intermediate clear areas formed in eggs in which movement, as judged by departure from the spherical form, had been only very slight.

TABLE II

The effect of various high hydrostatic pressures upon the amoeboid movement of *Arbacia* eggs. (Room temperature 24.3–26.5° C.)

Pressure		Movements	
lbs./in.^{2}	Atmospheres	Experimental eggs	Control eggs
5,000	340	None	Active movement
3,500*	238	None	Active movement
2,000	136	Some eggs slightly aspherical, but no visible movement.	Active movement
1,000	68	Some active movement.	Active movement

* These eggs were finally returned to atmospheric pressure, after which they became angular within six minutes, and amoeboid within fourteen minutes.

In another experiment unfertilized eggs were treated with urea, by mixing of drops as already described (page 83). Eggs from the same female were treated simultaneously in hydrogen and in carbon dioxide-free air. No movement took place in hydrogen, while active movement occurred in carbon dioxide-free air. The cover slips were then interchanged; and the eggs originally in carbon dioxide-free air stopped movement in pure hydrogen, while those originally in hydrogen began to move quite well in carbon dioxide-free air.

The Effect of High Hydrostatic Pressure on the Amoeboid Movement of Arbacia *Eggs*

In one experiment unfertilized urea-treated eggs were mounted in the pressure chamber, and left at atmospheric pressure until active movement was in progress. The pressure was then raised to 5,000 lbs/in.^{2} (340 atmospheres). Movement ceased in most of the eggs and was only slight in the remainder. Moreover, the outline of all of the eggs became less angular. Accordingly a series of experiments was undertaken in which unfertilized eggs were mounted in the pressure chamber as quickly as possible after treatment with urea, and raised immediately to the desired pressure. The results are shown in Table II. In control experiments urea-treated eggs were observed concurrently at atmospheric pressure.

The effects of high hydrostatic pressure on unfertilized eggs in sea water was investigated in one experiment. Eggs were compressed to 7,000 lbs/in.^{2} (476 atmospheres) for five minutes and then raised to 10,000 lbs/in.^{2} (680 atmospheres) for an additional five minutes, while under observation. The pressure was then rapidly released. There were no visible effects either while the eggs were under pressure or afterwards. Upon subsequent insemination 100 per cent of the eggs yielded good fertilization membranes.

DISCUSSION

The fact that cortical reaction and membrane elevation took place without oxygen in response to certain chemical and physical agents accords well with expectation. For in the experiments of E. B. Harvey (1930) membrane elevation took place in response to the action of sperm in the presence of only minute traces of oxygen, so long as there was sufficient oxygen for the sperm to swim. Indeed Harvey concludes that "If oxygen is necessary for membrane formation, it is an almost infinitesimal amount." Since it is now known that oxygen is not necessary for the egg cortex to respond, doubtless the only factor which prevents its response to sperm in the absence of oxygen is the immotility of the sperm. Additional evidence in this direction is supplied by Loeb's (1915*b*) experiments in which sperm immobilized by treatment with NaCN were incapable of fertilizing the eggs of the sea urchin *Strongylocentrotus purpuratus;* this effect was reversed when the sperm had recovered its motility.

It has been shown in our experiments that suitable chemical treatment under anoxic conditions may lead to cleavage after readmission of air. This eliminates any suggestion that oxygen is necessary for the cortical reaction if cleavage is to take place subsequently. However, the cortical reaction sets in motion processes for which oxygen is necessary. Thus in the absence of oxygen or at very low oxygen tensions no cleavage takes place (Loeb, 1895; E. B. Harvey, 1927; Amberson, 1928). Moreover, in sea urchin eggs the cortical reaction is followed by a striking increase in the rate of oxygen consumption; and this is true regardless of whether membrane elevation is initiated naturally or artificially (see for example Warburg, 1908, 1910; Loeb and Wasteneys, 1911, 1913).

The idea that cleavage furrow formation and amoeboid movement may be different manifestations of the same type of cellular activity has been expressed by Marsland (1936, 1938, 1939) and by Schechtman (1937). It is possible that the amoeboid movement of *Arbacia* eggs in urea solution may perhaps be regarded as a perversion of the activity which would normally be expressed in cleavage. While it seemed at first possible that the oxygen tensions which would just support cleavage might be of the same order of magnitude as those which would support amoeboid movement in *Arbacia* eggs, nevertheless our results show quite clearly that this is not so. Some degree of amoeboid movement occurred even at 0.42 mm. oxygen—a tension far too low for cleavage, although of the same order as that (0.3 mm. oxygen) which will just support some movement of the marine amoeba *Flabellula mira* (Kitching, 1939*b*). It must be remembered, in comparing amoeboid movement with cleavage, that the latter is usually dependent on certain nuclear changes, quite apart from those phenomena of cytoplasmic gelation, such as growth of the asters, which lead to furrow formation.

The influence of high hydrostatic pressure provides an interesting comparison between cleavage of inseminated *Arbacia* eggs (Marsland, 1938), amoeboid movement of *Amoeba dubia* and *Amoeba proteus* (Marsland and Brown, 1936), and amoeboid movement of unfertilized *Arbacia* eggs in urea solution (as recorded in this paper). The effective range of pressure is approximately the same in each case. At 340 atmospheres the ends of the pseudopods of amoebae become spherical, and the organisms finally (at 400 atmospheres) round up entirely; at such pressures inseminated *Arbacia* eggs do not cleave; and amoeboid *Arbacia* eggs stop moving and tend to round up. Amoebae stop moving at 250 atmospheres; unfertilized *Arbacia* eggs in urea solution do not lose their spherical form at 230 atmospheres; and cleavage of inseminated eggs is delayed at pressures below 333 atmospheres. The solation of the cortical gel, which is believed to account for the inhibition of cleavage of inseminated eggs and for stoppage of movement of amoebae under the influence of high hydrostatic pressures, no doubt also accounts for stoppage of amoeboid movement of unfertilized *Arbacia* eggs in urea solution.

It seems rather remarkable that pressures as high even as 680 atmospheres have no apparent harmful effects, at least as regards the cortical response, on the unfertilized eggs of *Arbacia punctulata,* when applied for short periods (5–10 minutes) of time.

SUMMARY

1. A special technique has been developed for transfer of sea urchin eggs (or other suitable objects) from one solution to another under anoxic conditions while under microscopical observation. The transfer is done with a fine glass loop in an anoxic micromanipulation chamber.

2. Unfertilized eggs of *Arbacia punctulata* were subjected in an atmosphere of pure hydrogen (and water vapor) to treatment with certain chemical and physical agents which in air are known to produce a cortical response, followed usually by membrane elevation.

3. A typical cortical response, usually followed by membrane elevation, was obtained in complete absence of oxygen by treatment with ¼ per cent saponin in sea water, isotonic urea solution, or isotonic sucrose solution, or by micro-puncture. The time relations were the same without oxygen as with it.

4. Transfer of unfertilized *Arbacia* eggs in urea solution, followed by several washes of sea water, all under anoxic conditions, led to cleavage after readmission of air. Thus the treatment which initiates cleavage does not require oxygen.

5. The amoeboid movement of unfertilized *Arbacia* eggs in isotonic urea solution was stopped reversibly in absence of oxygen. Cleavage also is known to require oxygen; but it was found that amoeboid movement took place at an oxygen tension well below that required for cleavage.

6. Unfertilized *Arbacia* eggs in urea solution immediately stopped all movement when the hydrostatic pressure was raised to 340 atmospheres. If the pressure was raised to 230 atmospheres or more before the eggs had begun to lose their spherical shape, no movement occurred. This inhibition of movement was reversed when the pressure was released.

7. Unfertilized eggs which had been compressed to 680 atmospheres for several minutes showed no ill effects, and when inseminated afterwards at atmospheric pressure gave good fertilization membranes.

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ACETYLCHOLINE AND NERVOUS INHIBITION IN THE HEART OF VENUS MERCENARIA

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During a study of the comparative pharmacology of the hearts of invertebrate animals the students in the physiology course at the Marine Biological Laboratory[2] have found the heart of the quahog, *Venus mercenaria,* to be extremely sensitive to acetylcholine. Jullien showed (see Jullien, 1936, for summary and references) that the hearts of several mollusks are inhibited by acetylcholine. He found that atropine does not antagonize this inhibition.

The Clam Heart as a Test Material for Acetylcholine

The sensitivity of the heart of *Venus* to acetylcholine is such and the preparation is so stable that it has some advantages over the commonly-used leech muscle as a test material for acetylcholine. The heart can be tested *in situ;* or the heart can be mounted in a small tube of sea water which can be replaced by the test fluid; or the heart can be mounted in a chamber through which test fluid is perfused. When test solutions are dripped on the heart *in situ* the threshold is many times higher than by the other methods. We have found the perfusion chamber method most satisfactory and have used several types of chamber. The fluid enters through a tube at the bottom and an overflow tube at the top is at the level of the surface of the water in a constant temperature bath. In some experiments the heart is mounted either by ligatures around the ends of the ventricle or by tiny glass hooks and the fluid surrounding the heart slowly changed from aspirator bottle reservoirs. In other experiments the heart is perfused directly in the chamber through a cannula which enters along the intestine into the ventricle and holds the heart at the lower end. The perfused hearts show a quicker response to test materials than those in circulating medium.

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[1] Abstracted in part by Prosser, C. L., and H. B. Prosser, 1937. The action of acetylcholine and of inhibitory nerves upon the heart of *Venus. Anat. Rec.,* **70,** suppl. 1 : 112.

[2] Some of the experiments reported here were first done in the Physiology Course at the Marine Biological Laboratory. I am grateful to all those students who have been interested in this problem during four summers. I also wish to acknowledge the help of Hazel B. Prosser in verifying and extending the experiments.

The beat of the heart is very sensitive to tension and to rate of flow. Frequently a heart fails to beat when first mounted, but when a slight tension is applied the beat starts regularly. It is very important to keep the tension and the rate of flow through and around the heart constant. Indeed Koehring (1937) has found that under natural conditions the heart of *Mya* does not beat when no fluid is flowing through the mantle cavity. In general, increasing the rate of flow increases the amplitude of the beat, decreasing the rate of flow diminishes the amplitude of beat. We normally count drops from the overflow of the chamber and keep the rate at approximately two drops per second.

The stability of the preparation depends partly upon the temperature. The sensitivity to acetylcholine declines slightly with time but this decline is much slower below 20° C. than above that temperature. We have frequently worked with preparations during a period of 24 hours or longer at 15–20° C. Normally a heart mounted in the morning retains high sensitivity throughout the day.

Sea water has ordinarily been used as a perfusion fluid, but in experiments done at an inland laboratory in the winter Van't Hoff's solution of artificial sea water has been found entirely satisfactory. Acetylcholine diluted in sea water retains its potency for four to six hours after which a decline in its activity can be observed.

The sensitivity of the hearts to acetylcholine and their stability as long-lasting preparations is much greater if the clams are freshly dug. Clams which have been kept either in running sea water or in a refrigerator for a week are much less satisfactory than those which have been dug within a day or two.

The sensitivity of the heart of *Venus* to acetylcholine shows seasonal variation. We have made tests at different times of the year during three years on over a hundred specimens. The sensitivity is highest in the spring. From March to early July marked inhibition occurs with dilutions as great as 10^{-12} to 10^{-11}. Occasional preparations show even greater sensitivity. Sensitivity can be increased slightly by eserinization. Late in the summer and during the fall and early winter the sensitivity is low and thresholds are of the order of 10^{-10} to 10^{-9}. This variation, both seasonal and among individual clams, is much greater than has been reported for the leech muscle which usually shows a threshold after eserinization of 10^{-9}.

The beat of the *Venus* heart usually ceases completely at a concentration ten times as great as that which slightly decreases the amplitude. The leech muscle gives differences in contraction over a concentration range of several logarithmic units, hence it is more useful for testing different concentrations of acetylcholine. The advantages of the *Venus* heart are its ease of preparation, its constancy of response over periods of many hours, its high sensitivity and resulting ability to detect traces of acetylcholine. The *Venus* heart provides material useful for acetylcholine bio-assay if attention is paid to the factors of tension, rate of perfusion, temperature, freshness of the preparation, age of the acetylcholine dilution, and season of the year.

The Effects of Drugs

Acetylcholine has primarily a negative inotropic action on the clam heart (Fig. 1) although frequently a slight slowing also occurs (Fig. 5, *A, B*). The threshold for the reduction in amplitude is slightly lower than that for the reduction in rate. When perfusion continues with a threshold concentration of acetylcholine, some recovery from the initial depression may occur. When higher concentrations are used the beat is stopped in diastole, a slight fall in tonus may occur, and no recovery occurs until the acetylcholine is removed.

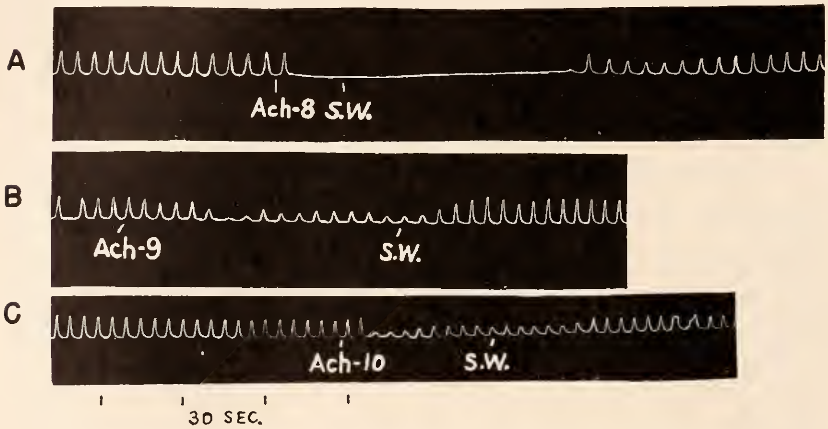

FIG. 1. *A,* effect upon a *Venus* heart in a chamber of acetylcholine 10^{-8} followed by sea water (S.W.). *B* and *C,* effects of acetylcholine 10^{-9} and 10^{-10} respectively. Late July preparation.

Previous treatment with eserine (10^{-4}) for a period of 15 to 30 minutes increases the magnitude of the acetylcholine effect (Fig. 5*B*). The sensitivity to acetylcholine may be increased several times but the most marked effect is the prolongation of the inhibition.

Jullien found that atropine makes the beat of snail and oyster hearts irregular. We have found atropine to be extremely toxic to the heart of *Venus.* In concentrations of 10^{-4} or 10^{-5} it not only makes the beat irregular but frequently stops the heart (Fig. 2, *A, B*). Preparations differ widely in their sensitivity to atropine. When non-toxic concentrations of atropine are used in combination with or preceding treatment with acetylcholine there is no consistent antagonism of the acetylcholine inhibition (Fig. 2, *C, D*). This must mean either that any possible antagonism is masked by the toxic effects or else that the receptor mechanism for acetylcholine inhibition is different from that in the vertebrate heart and other systems where atropine antagonism is found.

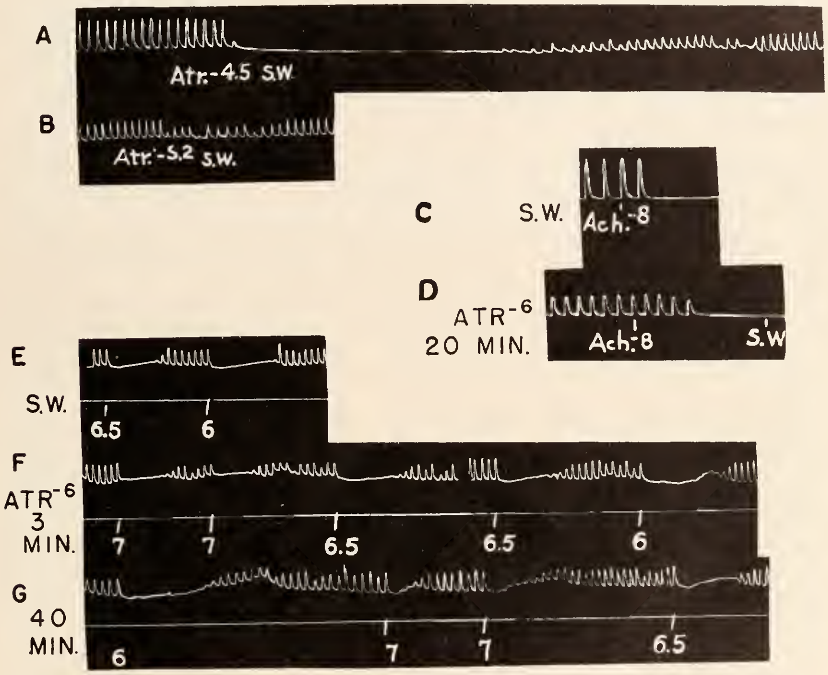

FIG. 2. Three experiments on the effect of atropine on the heart of *Venus*. *A,* toxic effect of atropine sulphate $10^{-4.5}$ followed by sea water. *B,* toxic effect of atropine sulphate $10^{-5.2}$ followed by sea water. *C,* another preparation, effect of acetylcholine 10^{-8} before and *D,* after bathing with a non-toxic concentration of atropine, showing lack of antagonism. The slight delay in inhibition after atropine is within the normal variation of sensitivity to acetylcholine. *E, F, G,* inhibition by visceral ganglion stimulation in another preparation. Numbers give position of secondary of inductorium. *E,* in sea water, *F,* after treatment with atropine sulphate 10^{-6} for 3 minutes and *G* for 40 minutes. Atropine 10^{-5} was found very toxic for this preparation.

Adrenaline increases the frequency of the heart beat and to a slight extent the tonus of the heart. In high concentrations it may cause cessation of beat in systole.

Nicotine acts much like acetylcholine in causing a reduction in amplitude and arrest in diastole.

Manner of Action of Acetylcholine on the Heart of Venus

Acetylcholine acts in opposite manner upon the hearts of mollusks and arthropods. In the mollusks its effect is always inhibitory as in the vertebrates. In the arthropods, however, it is an accelerator (cf. Welsh, 1939*a, b* on several decapods; Hamilton, 1939, on the grasshopper). The threshold for the accelerating action on arthropod hearts is higher than that for inhibition in the molluscan hearts.

Histological examination shows nerve cells in the hearts of all arthropods examined but in only a few mollusks (cf. Alexandrowicz, 1912). Miss Audrey Smith in the 1938 Physiology Class at the Marine Biological Laboratory has made a careful histological study of the hearts of the crab *Libinia* and the clam *Venus* by methylene blue, toluidin blue, and silver techniques. Her preparations show nerve cells in the spider crab heart and in the ganglia of the clam; they show nerve endings but no nerve cells in the heart of *Venus*.

When examined microscopically a weakly beating heart of *Venus* shows the contraction originating for successive beats at different points on the heart. It is difficult to see this in a strongly beating heart. These lines of evidence indicate, therefore, that the contraction of the heart of *Venus* is strictly myogenic in contrast to arthropod hearts and suggest that acetylcholine acts directly on heart muscle in this animal. In the heart muscle itself there must occur pacemaker, conduction and contraction processes.

We have examined microscopically the living hearts of clams before and after the beat is stopped by acetylcholine. When completely inhibited, a heart can readily be excited to single contractions by local mechanical or electrical stimulation. These contractions are sometimes localized and sometimes spread through the entire heart. This must mean that the contractile mechanism and sometimes the conductile mechanism also are intact. No rhythmic action potentials are present in an inhibited heart. During recovery from acetylcholine inhibition little beats may occur locally at scattered points over the heart, but they do not spread to surrounding regions. It is as if the pacemaker stimulus is either too weak to set up a general wave of excitation or else the wave is not conducted normally. The fact that the principal action is a negative inotropic one indicates an upset in conduction although the slight chronotropic action suggests an effect upon the pacemaker mechanism also. It seems likely, therefore, that acetylcholine acts either upon the pacemaker processes or the conduction mechanism or both rather than upon the mechanism of contraction.

Mechanism of Normal Cardiac Inhibition

It has been demonstrated by Carlson (1905) and Budington (1904) that stimulation of the visceral ganglion of the clam causes inhibition of the heart. The effect of stimulating at several different intensities is shown in Fig. 3*A*. The gills on the left side were cut and rigidly mounted electrodes were placed in contact with the visceral ganglion which lies on the inner side of the posterior adductor muscle. Thus the electrodes remained in constant position for many hours. Motley (1934) maintained that such depression of beat as he obtained with freshwater mussels on ganglionic stimulation might be due to movements of the foot and visceral mass. This is most certainly not true in our experiments since the inhibition occurs clearly when the heart is lifted up away from the visceral mass except at its two ends.

As Budington pointed out, the duration of the inhibition far outlasts the nerve stimulation and there may be cardiac escape if a weak stimulation continues. The threshold of this inhibition decreases slightly during the first half-hour after a clam is prepared. It then remains constant for many hours. Similar arrest of the heart occurs reflexly if the mantle or foot is pinched (Fig. 3*C*). If a series of tetanic stimuli are applied to the visceral ganglion at 20-second intervals the later bursts in the stimulation series are less effective than earlier ones at the same intensity (Fig. 3*B*). If, however, one or two minutes elapse between stimulation periods, the response remains very constant. If the inhibition be due to a liberated mediator this effect might be due to the requirement of time for resynthesis of the mediator in the nerve axon terminations.

The effect of acetylcholine upon the heart can very easily be duplicated in duration and magnitude by stimulating the visceral ganglion. It is possible, therefore, that acetylcholine acts as an inhibitory mediator here as it does in the vertebrates.

Jullien (1936) argued that because atropine does not antagonize the acetylcholine inhibition of the mollusk heart, a cholinergic inhibitory mechanism is probably not present. It seems to us unjustified to argue from drug action to nervous mechanism. Two tests must be applied to any theory of chemical mediation: the presence of the active substance should be demonstrated following nerve stimulation, and the effects of drugs should be combined with nerve stimulation.

Transfer of fluid from an intact clam heart subjected to nerve inhibition by visceral ganglion stimulation to another heart either in a small chamber or perfused *in situ* has been attempted in several experiments on each of eleven preparations. Direct perfusion of the inhibited heart is very difficult and it is best to fill the pericardium in which the punctured heart lies with sea water containing eserine. Intermittent stimulation of the visceral ganglion is then carried out for thirty seconds during which the heart is at a standstill. The two cubic centimeters of

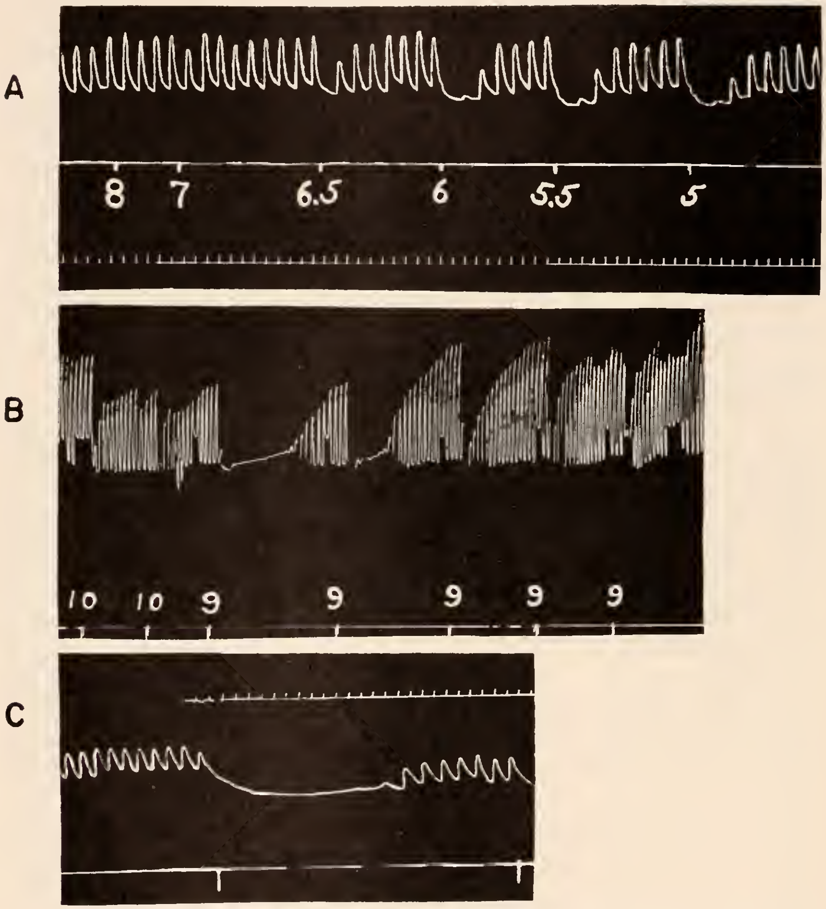

Fig. 3. *A*, effect of brief tetanic stimulation of visceral ganglion at intensities indicated by position in centimeters of inductorium secondary coil. *B*, effect of repeated frequent stimulation at same intensity (9 cm.). *C*, reflex inhibition; the mantle was pinched at time indicated by the signal. Time intervals in *A* and *C*, three seconds.

fluid bathing the heart are then transferred to the test heart. In some experiments there was no effect. Eight of the eleven preparations, however, showed some reduction in amplitude of beat of the test heart when bathed by fluid from an inhibited heart (Fig. 4*C*). When eserine was added to the sea water in the pericardium to prevent hydrolysis of any acetylcholine liberated and to the test heart for sensitization, the results were much more convincing than when no eserine was used. The amount of fluid that can be obtained from the pericardium and particularly the amount that can come in contact with the interior of the heart is very small. The results do, however, suggest that a substance is liberated in the normally inhibited heart, is protected by eserine, and acts upon a test heart much like acetylcholine.

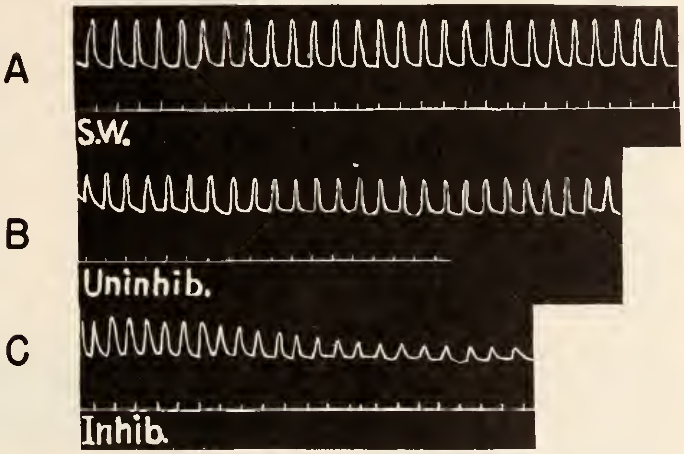

FIG. 4. The effect of fluid transferred from an *in situ* heart to a test heart in a small chamber. *A,* beat of test heart in sea water. *B,* transfer of eserinized sea water from pericardial cavity of heart beating normally. *C,* transfer of eserinized sea water from pericardium of same heart inhibited for 30 seconds by intermittent tetanic stimulation of the visceral ganglion. Time signal, 6 seconds.

In a series of experiments drugs were combined with the nerve inhibition. Atropine in a non-toxic concentration was allowed to drip over a heart which was being inhibited by visceral ganglion stimulation. No decrease in the inhibition was obtained during many minutes of treatment with atropine (Fig. 2, *E, F, G*). This agrees with the fact that atropine does not antagonize the acetylcholine inhibition (Fig. 2, *C, D*).

The effect of eserine upon the nerve inhibition is shown in Fig. 5. When the heart is bathed with eserine (10^{-4} to $10^{-3.5}$) for 15 to 30 minutes it shows not only greater acetylcholine inhibition (Fig. 5*B*) but also more nerve inhibition (Fig 5*D*). The threshold for the nerve inhibition is altered little if any but the duration of threshold inhibition is very greatly lengthened. In one experiment, for example, an inhibitory stimulus which before eserinization caused inhibition lasting approximately 30 seconds, after eserinization resulted in inhibition which lasted 50 minutes. Recovery from the effect of eserine is very slow; in these experiments it was appreciable after one-half hour but was not usually complete after several hours. These results indicate that the effect of eserine is not to sensitize the heart but to prevent destruction of the inhibitory mediator. If acetylcholine is the mediator involved its action should be greatly prolonged because its hydrolysis by choline esterase is retarded. This is found to be true.

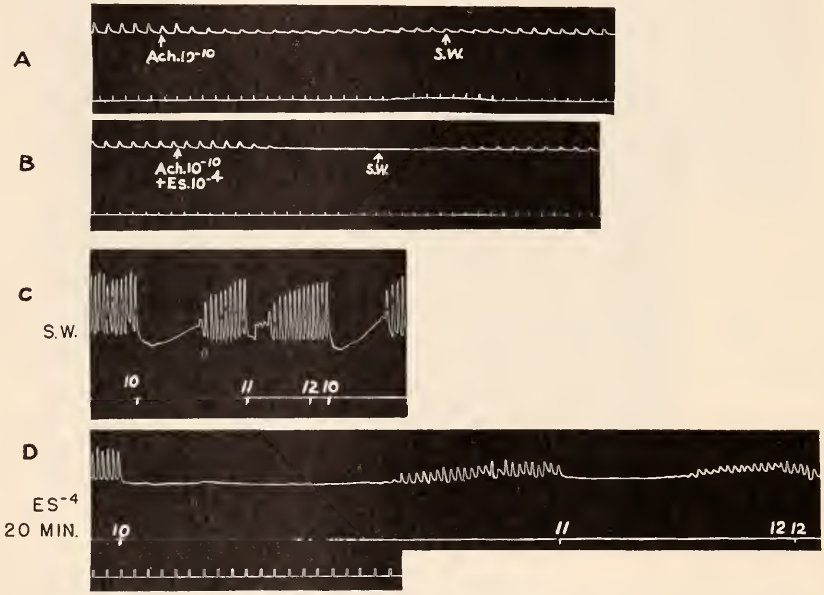

FIG. 5. Effect of eserine sulphate upon inhibition of the heart. *A* and *B* chamber preparation. *A*, inhibition by threshold concentration of acetylcholine 10^{-10}. *B*, inhibition due to same concentration of acetylcholine combined with eserine (10^{-4}) after eserinization for approximately 15 minutes. Eserine alone had no perceptible effect upon beat. *C* and *D in situ* preparation. *C*, inhibition of heart bathed with sea water by visceral ganglion stimulation at indicated positions of inductorium secondary. *D*, inhibition at same intensities of ganglion stimulation after 20 minutes of eserinization. Time interval in *A* and *B*, 3 seconds; in *C* and *D*, 10 seconds.

It is of interest that Bacq (1935) found a moderate amount of choline esterase in the blood of the pelecypod, *Pectunculus,* and in other molluscan tissues. He found several molluscan body muscles to respond to acetylcholine, but Bacq and Coppée (1937) failed to get potentiation by eserine of responses of the foot of *Buccinium* or of *Mya.* Jullien, Vincent, Brouchet, and Viulet (1938) found very low acetylcholine and esterase contents in lamellibranch as contrasted with pulmonate mollusks. The very high sensitivity to acetylcholine, and the marked prolongation of inhibition by eserine agree with the low cholinesterase content of the heart of *Venus* as reported by Smith and Glick (1939). The slow recovery from the eserine effect indicates that the reactivation of the small amount of cholinesterase present must be a very slow process.

In summary, acetylcholine and nerve inhibition give similar pictures in *Venus;* in some experiments fluid transferred from inhibited eserinized hearts arrests test hearts; and eserine greatly prolongs both the inhibition due to acetylcholine and that due to visceral ganglion stimulation. These facts indicate that the normal inhibition in the heart of *Venus* may be by way of acetylcholine liberated at the terminations of nerve fibers from the visceral ganglion.

Summary

The heart of *Venus* is sensitive to acetylcholine in dilutions of 10^{-12} during the spring and 10^{-9} during the fall. It is useful as a test material for acetylcholine assay.

Acetylcholine appears to leave the contracting mechanism intact and to act on the pacemaker and conducting mechanisms of this myogenic heart.

Stimulation of the visceral ganglion causes inhibition in diastole resembling the effect of acetylcholine.

Atropine is very toxic to the heart. In non-toxic concentrations it antagonizes neither the effect of acetylcholine nor of nerve inhibition.

Fluid from a heart inhibited by visceral ganglion stimulation often depresses the beat of an eserinized test heart.

Eserine prolongs the inhibition due to acetylcholine and that due to nerve stimulation. It appears likely that acetylcholine is liberated as the normal cardiac inhibitory agent in *Venus.*

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THE EFFECTS OF HYDROSTATIC PRESSURE UPON THE POLAR LOBE AND CLEAVAGE PATTERN IN THE CHAETOPTERUS EGG

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It was first shown by Marsland (1936) that a hydrostatic pressure of 400 atmospheres " reversed " the incompleted cleavage furrow of a dividing *Arbacia* egg. If the pressure was then released within a short period of time no furrow again appeared until time had elapsed for the normal formation of the second cleavage. At that time both the first and second cleavage furrows cut in almost simultaneously. Marsland (1936, 1938 and 1939*a*) interpreted the reversal of the incutting cleavage furrow as due to the liquefaction of the cortical gel. Pease and Marsland (1939) have confirmed this effect with eleven other eggs. In addition, cortical solation has been demonstrated in *Amoeba* (Marsland and Brown, 1936), in the tentacles of suctorian Protozoa (Kitching and Pease, 1939), and Marsland (1939*b*) has shown plasmagel solation in *Elodea.*

This apparently general effect of hydrostatic pressure upon cortical gels seemed applicable to a study of the formation and properties of the " polar lobe " apparatus present in some spirally cleaving eggs. The egg of *Chaetopterus pergamentaceus* Cuv. proved admirable material. The early cleavage has been described by Lillie (1906). The polar lobe, although relatively small, prominently appears just prior to the first and second cleavages. A small lobe appears before the third cleavage. The lobe persists until just after the cleavage furrows are completed. The first cleavage is very unequal, the second produces two equal *A* and *B* blastomeres, the slightly larger *C* cell, and the much larger *D* blastomere which contains most of the original lobe material. The third cleavage is equatorial and is very nearly equal. The inequality of the first two cleavages accentuates any irregularities in the cleavage pattern which result from pressure. The differential distribution of various granules, in addition to the position of the polar lobe and polar bodies, serve as markers of the normal egg axes and allowed an accurate determination of the cell origins in the 2-, 4-, and 8-cell stages irrespective of the cell size.

The method, in general, was to apply 270–470 atmospheres pressure to the eggs during the formation of the polar lobe, and before the cleavage furrow had cut entirely through the egg. The pressure apparatus has been briefly described by Pease and Kitching (1939), and was so constructed that material could be kept under constant microscopical observation while under pressure. Control eggs, kept in the bomb without pressure, showed no deleterious effects. Controls were also kept for each experiment and when these had completed cleavage, 3½–8 minutes after the pressure was applied to the experimental material, the pressure was released. The eggs so treated were fixed in the 2-, 4-, and 8-cell stages, and, in addition, series of photomicrographs were made at intervals while the eggs were in the bomb to supplement the preserved material.

RESULTS

It was found that a pressure of 220 atmospheres sufficed to suppress the formation of the polar lobe and block cleavage if the pressure was applied during the early stages of lobe formation and before the cleavage furrow had cut deeply. Higher pressures were necessary to reverse deeply cut furrows, and a pressure of 270 atmospheres caused the fully formed polar lobe and all but the deepest cut furrows to be withdrawn and the cell to round up.

After removal of the pressure, when the control eggs had completed cleavage, the eggs remained round until the time for the normal second cleavage to begin, plus a delay approximately equal to the time they were held under pressure. Then the polar lobe re-formed and the first cleavage furrow appeared. Most frequently the second cleavage furrow appeared almost simultaneously and the egg divided at once into four cells with the cleavage planes along the polar axis (Figs. 6–8, egg *A*). The third cleavage appeared equatorially after the proper

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EXPLANATION OF PLATE FIGURES

FIG. 1. Dividing *Chaetopterus* eggs just before the pressure was applied.
FIG. 2. Forty-five seconds after the application of 330 atmospheres pressure.
FIG. 3. After 2 minutes under pressure.
FIG. 4. After 4 minutes under pressure following which the pressure was released.
FIG. 5. After 3 minutes following the release of pressure.
FIG. 6. Twelve minutes later, the cleavage being about 5–6 minutes delayed.
FIG. 7. Three minutes later.
FIG. 8. One minute later.
FIG. 9. Thirteen minutes later.
FIG. 10. Three minutes later, the cleavage being about 6–8 minutes late.
FIG. 11. Four minutes later.
FIG. 12. Two minutes later.

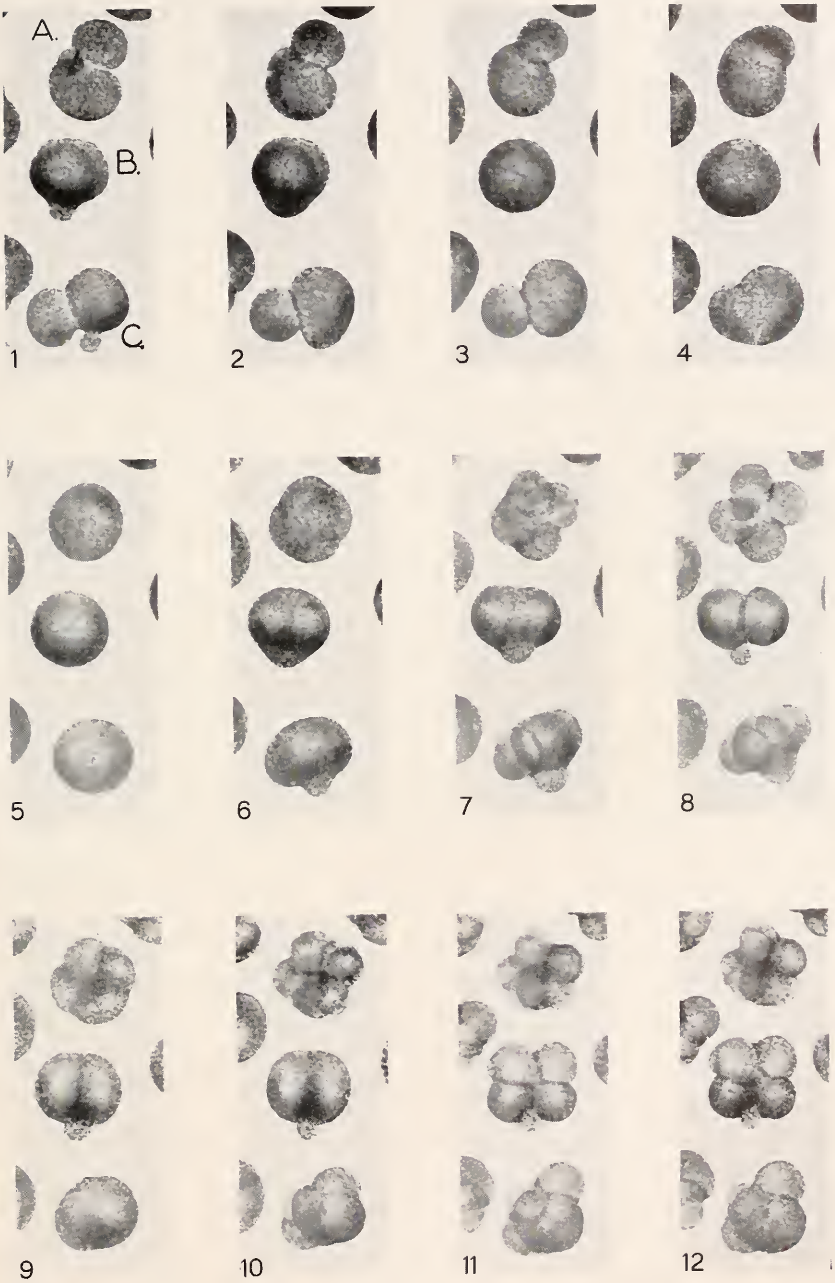
A.
B.
C.
1
2
3
4
5
6
7
8
9
10
11
12

Plate I

time interval. In these respects the *Chaetopterus* egg follows a similar sequence found by Marsland (1936) in the *Arbacia* egg.

When the first cleavage is blocked in this fashion (see Figs. 1–5 in the series of photographs) and the polar lobe re-forms at the time of the second cleavage (Figs. 6–8) it has the superficial characteristics of its *first* appearance during the first cleavage of the egg. Occasionally it is abnormally small or apparently lacking, but most commonly it is as large as the first lobe, or is abnormally large as in egg *C*, Figs. 7 and 8. It frequently has a tendency to pinch off as the cleavage furrows impinge upon it, and it may not be withdrawn into the *D* blastomere during or after cleavage. It may persist as a discrete body into the 8-cell stage as in egg *B* and the probability, under those circumstances, is that it has completely pinched off. Egg *A*, Fig. 8, shows a lobe that has persisted for an abnormally long time, but which was eventually withdrawn before the third cleavage.

As a result of blocking the first cleavage, the following cleavages are very frequently abnormal. A very common abnormality is a tendency towards equal cleavage shown by egg *A* in the series of photographs. Most frequently this is brought about by the pinching off of the polar lobe as the cleavage furrows of the first and second divisions impinge upon it simultaneously, but in other more rare cases the polar lobe material is apparently equally included in all four blastomeres.

In the same sense that the cleavage was sometimes abnormally equal, it was also found with widely different unequal abnormalities. The *AB* cell has exceptionally been seen larger than the *CD* cell. The first cleavage might be normally unequal, but the second might be abnormal, producing two equal large and two equal small cells, or the *A* and *B* blastomeres might be of different size, or the *C* cell abnormally large or small in relation to the *D* blastomere.

It is clear that the time relationships between nuclear and cytoplasmic division may be somewhat disturbed by pressure treatment because, instead of four cells being produced after the normal interval for the second cleavage, not uncommonly two cells are produced (egg *B*, Fig. 8), and in certain lots of eggs the majority divided into three cells (egg *C*, Fig. 8). Frequently the blastomeres of this latter type were approximately equal in size, and a study of the photographic series demonstrated that it was generally the *CD* cell which had undergone its second cleavage equally, while the *AB* blastomere remained undivided until the next division when 6-celled eggs were produced (Figs. 11 and 12, *C*).

The most interesting group of variations were those in which the first or second cleavage furrows were equatorial. A large percentage of this type occurred in an experiment in which the second cleavage was blocked by a pressure of 230 atmospheres applied for five minutes. At the time for the third cleavage many of these eggs were divided only by a second furrow in the equatorial plane. This phenomenon has also been exceptionally noted in eggs which were blocked only at the time of the first cleavage, and which divided only into two cells at the time of the second cleavage. This history is shown by egg *B* in the series of photographs reproduced.

In another experiment the eggs were completely blocked at the time of both the first and second cleavages by a pressure of 400 atmospheres applied for four minutes. The eggs had a tendency to become amoeboid, but the first completed cleavage of many of these eggs was equatorial and very much delayed. There was some evidence of partial furrows appearing and disappearing first in other regions of these eggs. There was, thus, considerable evidence that these eggs were multinucleate and several spindles could, in fact, sometimes be seen. The probable interpretation of all the cases in which the first or second cleavage furrow is equatorial is that these eggs have undergone two or one nuclear divisions without cytoplasmic cleavage. No cytological studies have been made and the exact relationships remain to be determined. Significantly, equatorial cleavage has never been observed before the time for the normal third cleavage.

It proved impossible to study these cleavage abnormalities in the later or trochophore stages. A negligible number of eggs subjected to pressure even became top swimmers. Complete degeneration occurred during the gastrula stage when the cells seemed to lack organization and proliferated extremely abnormally.

Conclusions

The withdrawal of the polar lobe and the rounding up of the cell under hydrostatic pressure is to be interpreted as due to the liquefaction or solation of the cortical gel which gives the lobe its structure. This is in accordance with the theory originally proposed by Marsland (1936, 1938 and 1939*a*). When the liquefaction has reduced the viscosity sufficiently surface tension forces round up the cell. The phenomenon is partly reversible and the polar lobe and cleavage furrows re-form for the following cleavages which, however, are characteristically abnormal.

Just as the cleavage furrow is able to form after centrifuging when the normal cytoplasmic constituents no longer underlie the cortex, so there is similar evidence leading to the belief that the underlying cytoplasmic elements play, at the most, a minor rôle in the formation of the polar lobe. Lillie (1906) first found that centrifuging did not hinder lobe formation in *Chaetopterus* and expressed the view that the phenomenon was essentially "ectoplasmic." Morgan (1935) was able to hold the *Ilyanassa* egg in the centrifuge in such a way that the animal pole lay at the centrifugal end, and the centripetal oil came to lie in the polar lobe region. Such eggs produced the lobe quite normally. As an extension of this, in connection with some other unpublished work, I have ultracentrifuged *Chaetopterus* eggs with forces of 120,000 and 170,000 gravities while they were rigidly held at random and supported in a gelatin gel. When removed from the gel and fertilized these eggs cleaved normally and examples could be found with the cleavage bearing any possible relation to the axis of stratification, and even the solidly packed oil layer might be included in the lobe.

That the mitotic apparatus is not an immediate essential for polar lobe formation was first demonstrated by Wilson (1904), who showed that alternate periods of form changes occurred in the isolated lobe of *Dentalium,* and this surface activity corresponded with the succeeding cleavages of the egg. Morgan (1933 and 1935) has made a much more extensive study of these form changes in the isolated *Ilyanassa* lobe, and he has emphasized that it is presumably a cortical effect. Wilson (1929 and 1930) divided the *Chaetopterus* egg with centrifugal force and found that only the fragments derived from the vegetative hemisphere produced polar lobes. These were quite normal in spite of the loss of cytoplasmic elements. Whitaker and Morgan (1930) demonstrated the normal formation of the polar lobe in vegetative fragments of the *Chaetopterus* egg cut with a micro-needle.

The close correlation of the lobe formation with the cytoplasmic cleavage process is further demonstrated by the work of Pasteels (1934). He found that eggs taken from the Mediterranean *Chaetopterus* very late in the season often do not cleave normally. Mitotic divisions are completed but the cleavage furrows only start to form and then reverse before completion. In these eggs the polar lobe also starts to form and then is withdrawn at the proper time intervals.

As a result of the hydrostatic pressure, it is also of interest that in the following cleavages the re-formation of the lobe shows irregularities that are probably to be homologized with the irregularities of the cleavages. This is particularly true apropos of the size of the re-formed lobe which may be quite variable. It is most frequently as large as the lobe normally formed for the first division, it commonly is larger, and infrequently smaller.

Marsland (1936, 1938, and 1939), working with the equally cleaving *Arbacia* egg, had unsatisfactory material for seeing irregularities in the early cleavage pattern. As a result of this study it becomes clear that the effects of pressure are not entirely reversible as far as the cell as a whole or the normal cleavage forces are concerned. Actually many of the abnormalities of cleavage resemble in many respects those obtained by Pasteels (1934) following ultraviolet radiation upon *Myzostoma* and *Aplysia* eggs.

SUMMARY

1. The polar lobe of the *Chaetopterus* egg is withdrawn and the cell rounds up under a hydrostatic pressure of 220 atmospheres if the pressure is applied in the early stages of lobe formation. A pressure of 270 atmospheres is necessary if the cleavage furrow is deeply cut.

2. This phenomenon is interpreted as due to the liquefaction of the cortical gel which gives the polar lobe its structure. Further evidence is added which leads to the belief that polar lobe formation is primarily a cortical effect of the vegetative pole region, to a very large degree independent of the underlying cytoplasm or the mitotic apparatus.

3. The suppression of the polar lobe and the cleavage furrow by pressure is partly reversible in that they re-form for the succeeding cleavages, but pronounced abnormalities in the cleavage pattern are characteristic. Typical irregularities are considered, including examples in which the first or second cleavage furrows have been produced equatorially.

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THE EFFECT OF SHAPE ON THE DEVELOPMENTAL AXIS OF THE FUCUS EGG[1]

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The cleavage plane of many cells has been determined by artificially imposed elongation. In general the long axis of the division figure comes to lie in the long axis of the cell so that the plane of division is perpendicular to the long axis. The plane and sequence of division of many eggs has been altered in this way, but the primary developmental axis, or polarity, has not been affected. Lindahl (1936) has found, however, that when sea urchin eggs are stretched by passing through capillaries, the first end to emerge tends to become ventral (if it is not the animal or vegetal pole) so that in this case the dorso-ventral axis is determined, as it also is by centrifuging (Runnström, 1926; Lindahl, 1936). In the *Fucus* egg, which appears to be more plastic than most animal eggs, the primary axis may be determined by centrifuging (Whitaker, 1937, 1938). The experiments to be reported were undertaken to test the effect of elongation on the division plane and the developmental polarity of the egg of *Fucus furcatus.*

METHOD

This species of *Fucus* is hermaphroditic and fertilization takes place when the egg capsule dissolves and releases 8 eggs into sea water in which motile sperm are present. The time of fertilization of the eggs from a given capsule can be observed quite precisely. Immediately following fertilization the naked egg secretes a film of soft gelatinous material which gradually hardens to form a rigid cell wall. If a recently fertilized egg is gently sucked into a glass pipette of less diameter than the egg, it enters and becomes elongated to an extent which depends on the relative diameters of the egg and the pipette. When it is gently blown out into sea water, the elongated shape is retained, no doubt largely because of the hardening of the cell wall in the elongated shape. Unless the egg is sucked into the pipette before the wall hardens, it will not enter.

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[1] This work has been supported in part by funds granted by the Rockefeller Foundation.

Only spherical eggs were selected so that they were taken into the pipette entirely at random with respect to any pre-existing organization which might occur in the egg. The eggs were taken into the pipette at 10–20 minutes after fertilization. Each egg was gently blown out into an individual 1 cc. syracuse dish containing either of two media: normal sea water at pH 7.8–8.2, or sea water acidified to pH 6.0 by adding 5 parts of McIlvaine's buffer (secondary sodium phosphate-citric acid) to 95 parts of sea water. The acidified sea water was equilibrated with atmospheric CO_2 tension by vigorous aëration through a large scintered glass nozzle until the pH was stable as indicated by a glass electrode. The long and short axis of each egg was measured by means of an ocular micrometer, and the ratio, long axis/short axis, serves as a measure of the degree of elongation. The individual dishes were placed in moist chambers. All stages of the experiments were carried out in the dark or in dim red light in a humid constant temperature room at 15 ± ¼° C., until the final results were recorded 15–24 hours after fertilization.

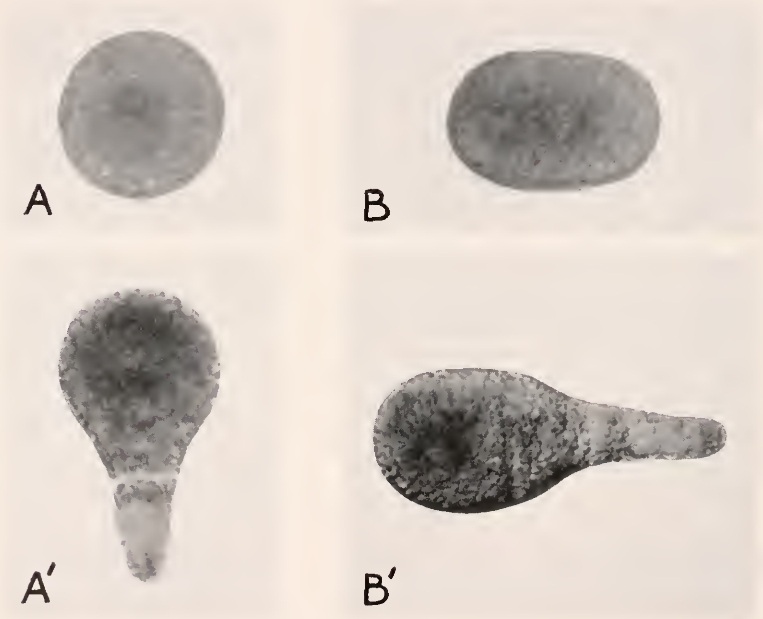

FIG. 1. Photomicrographs of *Fucus* eggs as seen from above. (*A*) Normal spherical egg soon after fertilization; (*A′*) similar egg after rhizoid has formed. (*B*) An egg which has been elongated soon after fertilization; (*B′*) an elongated egg which has developed in normal sea water at pH 8.0, showing typical formation of rhizoid at one end of long axis.

Experiments were carried out in June, July, and August, 1936, at the Hopkins Marine Station, and in March and April, 1938 at Stanford University.

Results and Conclusions

One hundred and fourteen eggs, elongated so that the ratio, long axis/short axis, ranged from 1.07 to 1.8, were reared in normal sea water. It was clear at once that a very high proportion formed the rhizoid very close to one end of the long axis. An example is shown in Fig. 1, *B′*. The shape of the elongated egg is very nearly that of a cylinder with rounded ends (Fig. 1, *B*). The angle between the long axis of the egg and the extrapolated axis of the rhizoid was taken as a measure of the proximity of the point of rhizoid origin to an end of the long axis. The rhizoid ordinarily grows out quite straight. Ninety-six per cent of the eggs formed rhizoids within 45° of an end of the long axis, and 53 per cent formed them within 10°.

When the eggs are divided into catagories based on the degree of elongation, the results show that the effect is more marked the greater the elongation. Thus of the 33 most elongated eggs (long axis/short axis = 1.4–1.8), 100 per cent formed rhizoids within 45°, and 73 per cent within 10°, of an end of the long axis.

Ordinarily the two ends of the elongated egg were quite similar in size and shape, and there was not a high correlation between the end which left the pipette first or last, and the end which formed the rhizoid. The relations were observed in 93 eggs, and 60 per cent formed rhizoids near the ends first to leave the pipette. It is not clear what determines which end of the long axis will form the rhizoid. Farmer and Williams (1898) note in their classical work on the cytology of *Fucus* that the centrosomes appear to arise from the egg protoplasm near the egg nucleus rather than from the sperm, as in the typical animal egg. They also note that the two asters, at either end of the egg nucleus, are often if not usually unequal in size in the early stage. The elongation of the egg undoubtedly orients the division figure in the long axis, and it is possible that the asymmetry of the early aster may have something to do with determining which end forms the rhizoid.

From these experiments it can be concluded that elongation of the *Fucus* egg determines not only the plane of cell division, but the axis of differentiation as well. The differentiation in fact precedes the cell division, since the rhizoid protuberance forms some hours before the first cell division.

Elongated Eggs Reared in Acidified Sea Water

Forty-seven eggs which were elongated in the same manner were reared individually in dishes containing sea water acidified to pH 6.0. In this case the degrees of elongation did not cover quite so great a range, the ratio, long axis/short axis, being 1.2–1.6.

In this medium the results are quite different. Seventy per cent formed rhizoids between 46° and 90° from an end of the long axis, i.e., formed rhizoids nearer an end of a short axis. An example is shown in Fig. 2, *A′*. Thirty per cent formed rhizoids within 45° and 13 per cent within 10° of an end of the long axis. The determination in this case is not so sharp as it is at pH 7.8–8.2, but it is clearly centered about the short instead of the long axis of the egg.

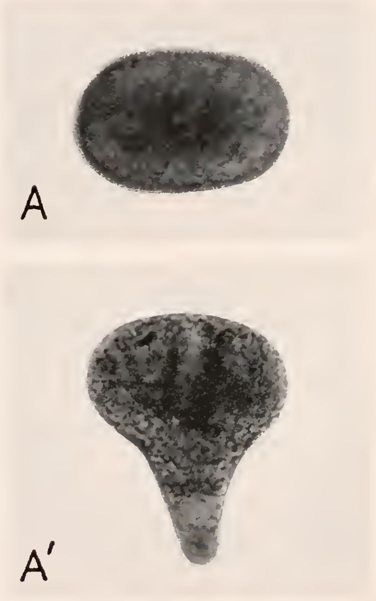

FIG. 2. Photomicrographs of *Fucus* eggs. (*A*) An egg which has been elongated soon after fertilization. (*A′*) Side view of a similar egg after developing in acidified sea water at pH 6.0, showing formation of rhizoid at end of short axis. The rhizoid formed toward the bottom of the culture dish.

It has been noted previously (Whitaker, 1937) that an egg developing on the bottom of a dish in medium at pH 6.0 tends very strongly to form the rhizoid toward the bottom of the dish, while at pH 8.0 the egg is more nearly indifferent to the bottom of the dish. There is now evidence that this phenomenon at pH 6.0 is largely due to the fact that the bottom of the dish is an impediment to diffusion, so that the egg is developing in a gradient of products diffusing from itself. When two eggs develop in close proximity they tend strongly to form rhizoids toward each other when the medium is at pH 6.0,[2] but not when it is at pH 8.0 (Whitaker, 1937; Whitaker and Lowrance, 1937). Acidity of the medium strongly intensifies the response of the eggs to their common diffusion pattern, and in like fashion it greatly intensifies the response of an egg to the bottom of the dish. All of the elongated eggs in medium at pH 6.0 formed rhizoids toward the bottom of the dish. Since an elongated egg naturally lies on the bottom of the dish with the long axis parallel to the bottom, a rhizoid forming toward the center of concentration of substances diffusing from the egg tends to form toward the lower end of the vertical short axis.

It appears highly probable that the response of the elongated eggs at pH 7.8–8.2 is essentially a shape effect, and that at pH 6.0 the shape effect is largely overcome or superseded by the response of the egg to the bottom of the dish. Some shape effect appears to persist as a resultant, to spread the positions of rhizoid origin, as noted. At pH 6.0, 13 per cent of the eggs still formed rhizoids within 10° of an end of the long axis, while at pH 7.8–8.2 no eggs formed rhizoids within 10° of an end of a short axis.

SUMMARY

1. When recently fertilized eggs of *Fucus furcatus* are gently sucked into a small pipette while the cell wall is hardening, and are then blown out into sea water, an elongated shape is retained.

2. Elongated eggs reared in normal sea water at pH 7.8–8.2 form rhizoids at or near one end of the long axis. The axis of differentiation, as well as the plane of cell division, is thus determined by the shape imposed on the cell. The exactness of determination increases with greater elongation of the egg.

3. When *Fucus* eggs develop in sea water acidified to pH 6.0, they acquire a very strong tendency to form rhizoids toward the bottom of the culture dish (which blocks diffusion to form a gradient of products from the egg). This tendency largely overcomes the shape effect when elongated eggs are reared at pH 6.0, and most of them form rhizoids toward the bottom of the dish near the lower end of the vertical short axis.

The author is indebted to Dr. E. W. Lowrance for assistance in carrying out the experiments.

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[2] Acidified with either citric acid-secondary sodium phosphate, or with HCl–$NaHCO_3$.

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PHYSIOLOGY OF REPRODUCTION OF OSTREA VIRGINICA

III. Stimulation of Spawning in the Male Oyster [1]

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It has been shown in the first part of the investigation concerning the physiology of reproduction of *O. virginica* (Galtsoff, 1938*a*) that the spawning reaction in the male consists in the emission of sperm from the testes into the cloaca and in its discharge from the organism by the outgoing respiratory current. The adductor muscle which plays such a conspicuous rôle in the spawning female is not involved in the sexual reaction of the male, for ejaculation is effected principally by the ciliary action of the epithelium of the genital ducts, cloaca and gills. Minute muscle fibres scattered in the walls of the gonoducts may act as constrictors preventing under certain conditions the release of ripe sperm, but they play no part in ejecting it from the testis. The male sexual reaction is also distinguished from that of the female by the absence of a refractory state. Ejaculation can be induced in the male a number of times until the organism is fatigued or the testis completely spent, whereas the female after each spawning passes through a refractory period lasting from two to five days during which it is not susceptible to sexual stimulation.

Stimulation of spawning in the male and the significance of male and female sexual reactions in the propagation of the species *O. virginica* are discussed in this concluding part of the investigation. Experimental technique used in this work has already been described in the first part of the study. The experiments were carried out during the summers of 1935–1938 at Woods Hole, Mass.

Stimulation by Temperature

That temperature has a stimulating effect on spawning of the male has been demonstrated by two sets of laboratory experiments in which the ripe males, taken from their natural environment, were placed in the laboratory tanks and kept at different constant temperatures not exceeding 30° C., or were subjected to a rapid rise of temperature to 32–38° C.

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[1] Published by permission of the U. S. Commissioner of Fisheries.

In carrying out these experiments oysters had to be observed continually for the discharge of sperm cannot be recorded with certainty on a kymograph or by some other mechanical device.

In the experiments summarized in Table I, ripe males kept in the bay or in the storage aquaria at temperatures indicated in column 3 were placed in the laboratory tanks at temperatures varying in different experiments from 25.6° to 30.0° C. Examination of the latent periods of the reactions given in the last column of the table discloses the presence of two distinct groups of oysters; those in which spawning took place after several hours of exposure (Exp. Nos. 4 and *7 I*) and a larger group of males in which the latent period varied from 25 to 65 minutes.

Table I

Stimulation of spawning by temperature

Exp. No.	Date	Temperature ° C.			
		Before	During	Increase	Latent period in minutes
3		16.0	25.6	9.6	33
4		18.8	25.7	6.9	695
58	July 9, 1927	17.5	29.0	11.5	60
58 a	July 9, 1927	17.5	29.0	11.5	65
313	July 1, 1931	20.0	30.0	10.0	*
392	July 11, 1932	21.0	27.5	6.5	32
10 I	June 20, 1936	20.0	28.0	8.0	25
7 I	July 3, 1936	20.0	28.0	8.0	620

* No reaction during 5 hours; spawned within 9 minutes after temperature was raised to 32.5°.

It is of interest to note that a male which fails to respond to a substantial rise of temperature may still be induced to spawn if the temperature is increased further. Thus, in experiment 313, Table I, the oyster subjected to 10° rise and kept for 5 hours at 30° failed to react but spawned within 9 minutes after the water was warmed at 32.5° C.

In a second series of experiments male oysters were placed in water the temperature of which was quickly raised by using an electric immersion heater and stirrer. Time elapsed between the moment the heat was turned on and the beginning of spawning is designated as a latent period of the reaction. In most cases heating was completed in 10 or 15 minutes. In several instances, however, males began to spawn before the desired temperature had been reached. In these cases heating was immediately discontinued. The data, obtained during the summers of

1937 and 1938, are summarized in Table II. For the sake of convenience, the results are grouped according to the temperatures at which spawning took place. Under the conditions of these experiments latent periods varied from 10 to 236 minutes, with the average values in different classes ranging from 16 to 39. Out of 94 cases, only in two, both occurring in the class 34–34.9° C., did the latent period exceed 100 minutes. If these two observations are disregarded, the average duration of the latent period of this class would be reduced to 30 minutes. No definite correlation between the latent period of spawning and the degrees of temperature can be noticed in these experiments.

Not all ripe males respond to thermic stimulation. As a matter of fact, in the experiments carried out during the summers of 1937 and 1938 with five and six-year-old males, only 59.7 and 42.1 per cent respectively responded to this stimulus. Histological examination disclosed that only a few of the males that failed to spawn had undeveloped gonads or had already completely discharged their sperm. In the great majority of cases the testes were found to be ripe, the lumens of the follicles and some of the ducts being filled with mature spermatozoa. Since these oysters were morphologically ripe, an explanation of their failure to spawn should be looked for in their physiological conditions, which are, however, but little understood. Realization of spawning in some of these specimens probably required stronger stimulus (higher temperature) than was used in the experiment. Experiment 313 (Table I) described above corroborates this explanation.

TABLE II

Latent periods (in minutes) of spawning reactions stimulated by rapid rise of temperature

Temperature, ° C.		Latent periods in minutes			Number of Observations
Before spawning	During spawning	Maximum	Minimum	Average	
20.0–20.7	28.0–29.9	18	12	16	3
18.4–21.8	30.0–30.9	47	11	34	3
18.6–21.4	31.0–31.9	40	10	27	7
18.6–22.0	32.0–32.9	37	11	22	16
18.0–22.0	33.0–33.9	64	14	23	25
18.0–22.0	34.0–34.9	236	10	39	35
19.0	35.0	63			1
18.7–22.0	36.0	48	26		2
18.0	38.0	55	53		2

The experiments just reported show very cearly that there is no definite critical temperature which would exercise a trigger-like effect and cause the spawning of all ripe males. As the evidence shows, both the exposure time and the effective temperature greatly vary. Long latent periods observed in several instances can probably be explained by the absence of free spermatozoa in the posterior part of the testis. Spermatozoa set free in the anterior part of the gonad must pass a considerable distance along the genital ducts until they reach the gonopore. It is probable that temperature speeds up this passage and that their accumulation near the gonopore stimulates the release mechanism. This assumption is, however, difficult to verify experimentally.

As will be shown below, spawning in some of the males can be induced only by a combined action of thermic and chemical stimuli. This probably accounts for the failure of a large percentage of males to respond to thermic stimulation alone.

Stimulation by Eggs

Since ripe females are easily stimulated to spawn by the presence of sperm in the water (Galtsoff, 1938*b*), it is natural to expect that eggs produce similar effect in ripe males. This expectation has been confirmed experimentally. The procedure employed in the present studies consisted in preparing a standard suspension (0.5 gram ripe ovarian tissue in 100 cc. of filtered sea water) and introducing a measured amount of it by means of a pipette into the pallial cavity of the male. The temperature of the suspension was adjusted to that of the water in which the male was kept and mechanical stimulation was carefully avoided by permitting the liquid to be drawn in to the pallial cavity by the ingoing respiratory current. Under these conditions the male was not disturbed and the mechanical stimulation of the mantle and of the adductor muscle was avoided. The time elapsed between the entrance of the first drop of the egg suspension into the pallial cavity and the emission of the first batch of sperm from the cloaca was measured with a stop-watch and designated as a latent period. In repeating the reaction, egg suspension was added at different zones of the pallial cavity starting from the anterior part and ending with the region adjacent to the cloaca. This was necessary in order to avoid the fatigue which was observed to develop after a repeated stimulation of one zone. The experiments were carried out only with the males which kept their valves wide open and were actively pumping water.

The observed latent periods must be slightly in excess of their true values since the exact time the stimulant acted upon the nervous receptors is not known and the appearance of sperm in the cloacal current is a fraction of a second later than its emergence from the gonopore.

Since this slight error occurs in all the observations it does not invalidate their results.

The latent periods of the spawning reaction induced by egg suspension varied from 3 to 120 seconds, depending upon the conditions of the male and the temperature of the water. In the majority of the cases they fluctuated between 5 and 8 seconds. The reaction to the first stimulation was often, but not always, slower than the subsequent responses, as can be seen from the following excerpts of the records of the experiments (Table III). Similarly, increased latent periods were noticed at the end of the experiment when the male appeared to be fatigued or almost completely spent.

TABLE III

Latent period (in seconds) of male spawning stimulated by egg suspension (— indicates no response)

Exper. No.	Time	Temperature	Latent Period
		° C.	*seconds*
419	10.25	25.5	29.8
	10.27	25.5	10.0
	10.30	25.5	9.2
413	11.16	24.8	21.0
	11.17	24.8	—
	11.19	24.8	8.5
	11.36	24.8	7.3
	11.42	24.8	7.0
	11.50	24.8	15.0
	12.50	24.8	—

The amount of sperm released and the duration of ejaculation greatly varied depending upon the state of ripeness of the male and the strength of the stimulus employed. In the majority of the cases ejaculation induced by a single dose lasted from a few seconds to several minutes. In some of the males, however, the first stimulation provoked copious ejaculation which continued for two or even three hours and stopped only when the supply of sperm in the gonad was exhausted.

That the reaction time is dependent on temperature was demonstrated by the experiments in which a single male was stimulated to spawn at gradually decreasing or increasing temperatures. The temperature was lowered by placing several beakers filled with ice and brine in the tank and stirring the water. At least four minutes were allowed for the oyster to acquire the temperature of the surrounding medium before egg suspension, kept in the same tank in a separate small vessel, was administered. Complete record of the experiment, presented in Table IV, shows that no ejaculation took place at temperatures below 12° C. This result is in accord with another experiment, not shown in Table IV, in which egg water (filtered egg suspension) was used.

By plotting the reciprocals of the latent periods recorded in these two experiments against the temperature, one can observe the straight line relationship between the two variables (Fig. 1). In drawing the lower curve (eggs), two observations had to be disregarded. One, at 12°, was obtained when the organism had not yet fully recovered from the exposure to low temperature (see Table IV). The second one, at 18°, the last one in the experiment, was made when the oyster showed obvious signs of fatigue.

TABLE IV

Effect of temperature on male spawning induced by eggs

Time	Temperature ° C.	Latent period in seconds	Duration of spawning in seconds	Remarks
3.00	20.0	9	190	Copious discharge
3.30	19.5	*	—	Occasional ejaculation
3.45	15.0	*	—	Ejaculation stopped
3.55	14.0	22	120	Copious discharge
4.10	10.5	—	—	No reaction
4.17	9.5	—	—	No reaction
4.26	9.0	—	—	No reaction
4.30	11.5	—	—	No reaction
4.37	12.0	—	—	No reaction
4.41	12.0	120	?	Light discharge
4.44	12.0	39	100	Light discharge
4.50	13.0	27.5	134	Copious discharge
4.56	14.0	18	77	Copious discharge
5.02	18.0	—	—	No reaction
5.05	18.0	17	?	Very light discharge
5.10	20.0	†		

* Stimulation omitted.

† Oyster not reacting; experiment discontinued.

The lowest temperature at which oyster population was observed to spawn in nature is about 17° C. (Loosanoff, 1939) or 5° higher than the lower limit of male spawning reaction recorded in the laboratory. It is doubtful that the males' ability to respond to chemical stimulation at 12° and shed sperm in this cold water has significance for the propagation of the species for it appears improbable that fertilizable ova can be present in water at this temperature.

It would be erroneous to infer that at 12° C. eggs would always induce spawning in ripe males. Numerous observations indicate that the lower temperature level of the male spawning reaction greatly varies in individual oysters. The following experiment illustrates this point. On August 1, at 9 A.M. three male oysters were placed in experimental tanks in water of 22.6° where they immediately opened and began to feed. Egg suspension introduced seven times in succession into their pallial cavities produced no reaction. At 9.30 A.M. the temperature was raised to 25.0° and the same egg suspension was again administered. This time it induced copious ejaculation, the latent periods varying between 8 and 14 seconds. These experiments clearly indicate that chemical stimulation alone is not always effective and that a combined action of increased temperature and chemical stimulus is often necessary to in-

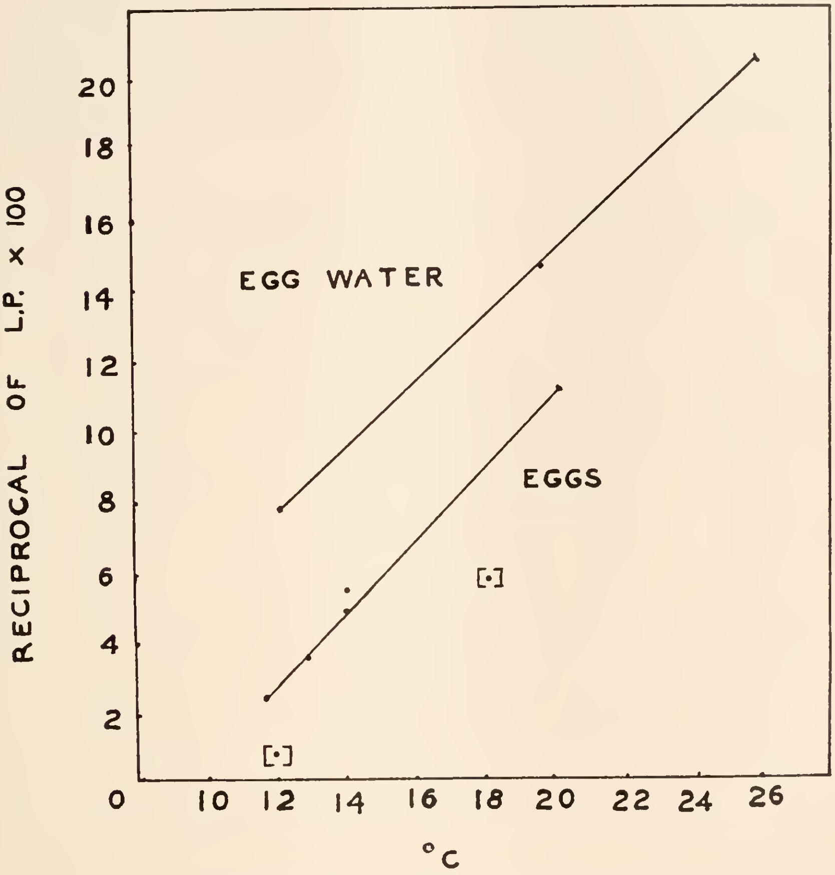

FIG. 1. Effect of temperature on latent period of spawning of the male oyster induced by egg suspension and egg water.

duce spawning. They also show that there is no definite single temperature level necessary for the realization of spawning in all ripe males of a given population.

Active Principle of Egg Suspension

The next step consisted in determining whether the eggs or the liquid phase of egg suspension has stimulating effect upon the male. The egg suspension used in these experiments was prepared by shaking and stirring 1.0 gram of ripe ovary tissue in 100 ml. of filtered sea water and permitting it to stand for 2 hours at room temperature.

Table V

Stimulation of spawning by eggs, and by liquid phase of egg suspension

Time	Temperature ° C.	Stimulus	Reaction	Latent period in seconds
P.M.				
2.00	22.2	Egg suspension	+	10.1
2.05	22.2	Paper filtrate	+	9.8
2.10	22.2	Paper filtrate	−	
2.18	22.2	Residue	+	11.5
2.19	22.2	Membrane filtrate	+	10.0
2.22	22.2	Membrane filtrate	+	9.2
2.23	22.2	Membrane filtrate	−	
2.25	22.3	Paper filtrate	−	
2.26	22.3	Egg suspension	+	12.7
2.28	22.3	Membrane filtrate	+	9.5
2.30	22.3	Paper filtrate	+	9.8
2.53	22.3	Paper filtrate	−	
2.55	22.3	Egg suspension	−	
3.30	22.0	Egg suspension	+	23.0
3.32	22.0	Membrane filtrate	+	11.7
4.10	22.0	Membrane filtrate	+	11.7
4.16	22.0	Membrane filtrate	+	14.3

Then the suspension was violently stirred and half of it filtered either through a double layer of No. 2 Whatman filter paper or through an ultrafine collodion membrane, capable of retaining Congo red particles. In both cases a perfectly clear liquid was obtained. The stimulating power of both filtrates, residue, and of unfiltered suspension were then tested on the same male. The results of numerous experiments consistently showed that both the eggs and the liquid phase of the suspension are equally effective. The record of one of these experiments, presented in Table V, indicates very clearly that the active substance passes through filter paper and through the collodion membrane without any loss of efficiency.

That the stimulating substance is a water-soluble product of egg secretion and is not derived from the ovarian tissue cells or juices which may be present in suspension, is proved by experiments with washed eggs. By repeated stirring in filtered sea water and decantations, eggs were thoroughly washed and set aside in a small amount of liquid. The supernatant fluid collected after a few minutes of standing, and filtered through paper was found to have a strong stimulating effect. In another set of experiments unfertilized eggs, discharged by a female which was stimulated to spawn by an increase in temperature, were collected, thoroughly washed, and found to be effective in stimulating spawning of the male.

The active principle of egg suspension was found to be unaffected by heating. It can even withstand boiling. Warming for 18 minutes at 46° produced cytolysis of eggs and slight opalescence of the liquid.

TABLE VI

Ejaculation stimulated by heated egg suspension

Type of suspension	Temperature ° C.	Latent periods in seconds	Number of tests
Normal egg suspension.	19.7	8 –13.5	5
Heated 18 min. at 46°...	19.8	9 –14.0	9
Heated 10 min. at 68°...	19.8	8.5–13.0	3
Boiled 30 seconds......	19.8	7.5–10.0	3

Heating at 67° or boiling, caused the formation of white precipitate. Each sample of suspension, after heating, was cooled, filtered and tested on 3 males. As one can notice by examining Table VI, there was no loss of potency due to heating and the stimulating power of a boiled sample was even slightly increased. From experimental evidence an inference can be made that, contrary to the condition found in the sperm suspension (Galtsoff, 1938*b*), the active principle of egg suspension is water-soluble and thermostable.

Stimulation by Foreign Eggs

Male oysters display no specificity in their spawning reaction and can be stimulated by eggs of other Pelecypoda and echinoderms. In carrying out these experiments (Table VII) the eggs of the following mollusks were tested: *Mytilus edulis, Mya arenaria, Venus mercenaria* and *Pecten irradians*. Positive results were also obtained with the eggs of *Asterias forbesi,* but *Arbacia* eggs were found ineffective. In each of these experiments a fresh male was alternately tested ten times with foreign and oyster eggs (controls). Each time 1 ml. of egg suspension, made of 0.5 g. of ovarian tissue in 100 ml. of filtered sea water, was used.

Since there is a considerable variation in the responsiveness of the males to stimulation depending on their readiness to ejaculate, the latent periods shown in the first column of Table VII can not be directly compared with one another. To judge the relative efficiency of the stimulus a comparison must be made with the values obtained for the stimulation of the same male with the oyster eggs. By examining these data presented in the table, one can notice that with the exception of *Pecten,* the stimulating power of various foreign eggs is generally weaker than that of the oyster eggs. The lowest values were obtained with the eggs of *Mytilus edulis* and *Asterias forbesi.*

TABLE VII

Comparison of the latent periods of ejaculation induced by foreign eggs and by oyster eggs (control). Temperature, 24–25° C.

Spawning of *O. virginica* induced by eggs of:	Latent Period in Seconds	
	Experiment	Control
Pecten irradians	4.6– 4.8	6.2
Venus mercenaria	8.0– 9.6	6.0– 8.0
Mya arenaria	8.0–30.0	6.0–14.0
Mytilus edulis	12.0–28.0	6.0– 8.0
Asterias forbesi	10.0–20.6	6.0– 6.2

Stimulation of Spawning by Hormones and Various Chemicals

The non-specificity of the male spawning reaction suggested the possibility that the shedding of sperm could also be induced by various chemicals. The correctness of this assumption was demonstrated in a series of tests summarized in Table VIII. All the males used were ripe and capable of spawning at the temperatures maintained during the experiments. The latent periods of ejaculation induced in them by oyster eggs varied between 5.2 and 6.2 seconds.

Out of 18 various substances which gave positive results, desiccated thyroid gland of sheep (thyroidin, Merck) proved to be the most effective. As a matter of fact, the reliability of this preparation in inducing ejaculation was so great that its use as a stimulant was considered preferable to that of oyster eggs and was adopted as a standard technique in our laboratory.

The stimulating action of thyroidin is primarily due to thyroxin, which in a pure form exercises a very strong effect on the male and causes copious ejaculation (Table VIII). The effect of theelin is much less pronounced, the latent periods being longer and the ensuing discharge of sperm is very light. Of other substances tested, the effect of glutathione is of special interest for in concentrations of 0.4 and 0.2 per cent, it irritates the adductor muscle, causing its contractions, and at the same time induces copious ejaculation, conditions not observed in any other case. The weakest response was recorded with peptone, egg albumen and some of the sugars.

TABLE VIII

Chemical stimulation of spawning in male oysters. Temperature 23°–25° C.
(Unless otherwise indicated all solutions are shown in grams per mls. of sea water.)

Substance	Number of Tests		Latent periods in seconds
	Positive	Negative	
Thyroidin (Merck) 0.25 : 50	69	61	3.0–29.0
Thyroxin (crystal, Squibbs) 0.4 mg. in 5.0 ml.	3		8.0–30.0
Theelin (Parke & Davis) (1 ml. containing 50 rat units)	2	4	9.0–65.0
Corpus luteum (desiccated) 0.25 : 50	3	4	8.0–13.8
Anterior pituitary, desiccated, 0.25 : 50	7	5	7.0–13.4
Posterior pituitary, desiccated, 0.25 : 50	1	1	11.0
Thymus (desiccated) 0.25 : 50	2	2	5.6–15.0
Adrenalin (Parke & Davis) 1.0 : 10,000	3	1	8.4–9.0
Cysteine, 0.1 : 25	6	4	13.0–17.0
Glutathione, 0.1 : 25	2	3	21.0
Glutathione, 0.05 : 25	1		14.0
Peptone, 0.25 : 50	4	10	5.6–11.0
Egg albumen, 0.25 : 50	5	16	9.0–16.0
Urea, 0.25 : 50	1	2	9.2
Yeast (Fleishman) 0.5 : 50	2		11.0
Dextrose, 4 per cent	1	1	10.2
Maltose, 0.8 per cent		2	
Maltose, 1.6 per cent	2		15.0; 12.2
Maltose, 2.0 per cent	2	1	14.2–15.0
Maltose, 4.0 per cent	2		10.0; 13.0
d. Arabinose, 0.8 per cent	1		12.4
d. Arabinose, 4.0 per cent	2		10.0–15.0
Starch, 4.0 per cent	2		15.0; 29.0

The following substances gave negative results: desiccated orchic substance; desiccated oyster meat; oyster blood; glycine; cysteine; cholesterine; yohimbine; cumarin; various inorganic salts which enter into a composition of sea water; 0.05 N hydrochloric and sulfuric acids.

There is no doubt that other compounds not yet tested may have a stimulating effect. For instance, Miyazaki (1938) reports that green algae, *Ulva pertusa,* and several species of *Entermorpha* and *Monostroma* sp. contain substances which induce spawning in the male *O. gigas.* From a biological point of view the stimulating action of sugars and starch is of special interest, for it appears feasible that these substances present in marine algae may under certain conditions be released in sea water and carried into the pallial cavity of the oyster.

Stimulation by Sperm

The fact that ejaculation in *O. virginica* may be provoked by a great number of organic substances suggests that the sperm of the oyster may also exercise this effect on a male. In testing this possibility no positive reaction was observed to take place within the latent periods of a few seconds, characteristic for chemical stimulation of male spawning. It was noticed, however, that some of the males spawned a few minutes after the addition of sperm. Since the experiments were carried out at slightly raised temperature, the spawning was first attributed to the latter factor. With the increased number of observations the explanation became untenable. In a series of more carefully conducted experiments, prior to the addition of sperm, ripe males were kept for a long period of time in warmed water (Table IX). Exposures lasting from 50 to 690 minutes at various temperatures, shown in column 4 of the table, were ineffective in inducing spawning of these males but each of them spawned within a few minutes following the addition of sperm (column 6). In several instances (Expts. 55, 227, 236, 265) the temperature did not exceed 23.5° and the actual rise of it was less than two degrees, conditions which usually do not stimulate ejaculation.

Even more convincing evidence is provided by Experiment 314. During four consecutive days this male was daily taken from the running sea water of 18.7–18.8° in which it was kept during the night and placed in an experimental tank. The temperature was then raised and maintained for several hours at 30.5°. Late in the evening the male was returned to its normal environment. No spawning occurred during the time the animal was kept under observation in warm water. On the fifth day as the water temperature reached 30.5° active sperm was added and ejaculation ensued within 8 minutes. It appears highly improbable that this was merely a coincidence.

As far as the author was able to ascertain, stimulation of male spawning by the sperm was recorded previously only for *Strongylocen-*

trotus lividus. Fox (1924) found that ejaculation in this sea urchin stimulates males as well as females to spawn.

It appears significant that the latent periods of the male and female spawnings stimulated by the sperm are of the same order of magnitude, greatly differing from the latent periods of male spawning stimulated by egg water and various chemicals. Apparently two distinct processes are involved. Stimulation of ejaculation by water-soluble substances acts on special receptors and involves the operation of a neuro-muscular mechanism which reacts within a few seconds upon receiving the stimulus. On the other hand, the active principle of sperm, insoluble in water, requires several minutes to initiate the response of the organism. There is a possibility that to become effective it must be absorbed through the digestive canal or by the gills, although direct evidence corroborating this assumption is lacking (Galtsoff, 1938*b*).

TABLE IX

Stimulation of male spawning by sperm

Exper. No.	Date	Initial temp. ° C.	Spawning temp. ° C.	Time of exposure in minutes	Sperm added. Latent period in minutes
27	July 23	20.8	24.3	50	9
55	July 7	20.6	21.0	220	27
126	July 12	20.6	30.5	130	6
227	July 9	22.0	23.0	60	12
236	July 10	22.0	23.5	60	20
265	July 17	21.5	23.0	120	12
291	July 23	21.0	23.8	53	9
296	July 29	21.0	24.0	66	11
305	July 26	21.0	24.0	58	14
310	June 30	19.4	30.0	58	10
314	July 2	18.7	30.5	440	*
	July 3	18.7	30.5	360	*
	July 4	18.8	30.5	560	*
	July 5	18.8	30.5	690	*
	July 6	18.8	30.5	5	8

* No sperm added; no reaction.

Relation between Ejaculation and Sensory Stimulation by Chemicals

The latent periods of spawning induced by various organic compounds are of the same order of magnitude as is the response of the oyster to sensory stimulation by chemicals (Hopkins, 1932*a*, *b*). Since tentacles situated along the free edges of the pallial folds are the first to come in contact with any substance brought into the pallial cavity by the ingoing current, it was thought possible that they are the bearers of the receptors involved in spawning.

To study this possibility Hopkins' method (1932*b*) was used. In brief, the procedure is as follows: the oyster is prepared by cutting off a posterior piece of shell, thus exposing the mantle. Then the tentacles are stimulated by adding the solution to be tested through a constant level tank, and the time elapsed between the addition of stimulant and the contraction of the tentacle is recorded. This method eliminates or at least greatly minimizes mechanical stimulation. It presents a great advantage in that the oyster is kept in running water and the chemicals used in the experiment are quickly washed away.

TABLE X

Sensory stimulation of the mantle and ejaculation induced by egg water. Temperature, 22–23° C.

Time	Percentage egg water	Latent Periods in Seconds		Duration of ejaculation
		Mantle	Ejaculation	
10–11 A.M.	1	19.5*	—	
11.30–12.10	10	5.5†	—	
1.25 P.M.	30	4.5	—	
1.35	30	4.5	—	
1.45	30	4.1	—	
1.55	30	3.8	49.5	80 seconds
2.00	30	4.3	11.5	2 hours
4.20	50	5.1	10.5	50 seconds
4.34	50	7.0	15.5	91 seconds

* Average of six readings.
† Average of four readings.
— No reaction.

Two sets of experiments were made using egg water (filtered standard egg suspension) and filtered thyroidin solution. The solutions were always freshly prepared and were kept at the same temperature as the water surrounding the male.

Three distinct reactions are involved in the response of the organism to chemical stimuli. A very light irritation causes a slight contraction of one or a group of tentacles; stronger concentrations of the same chemical may provoke a contraction of muscle fibres in the adjacent portion of the mantle and still stronger irritants cause the contraction of the adductor muscle. The three reactions do not always follow this order and occasionally one of them is missing. Sometimes the conditions are complicated by the occurrence of a second reaction of the mantle involving the contraction of the larger group of muscle fibres. In the experiments with egg water the reaction of tentacles was so indistinct that no determination of the latent periods could be made. The contraction of the mantle was, however, quite noticeable and the effect was observable even in dilutions of 1 per cent of standard egg water. The results of one experiment presented in Table X show that the mantle reacted to the concentrations of stimulant which were not strong enough to induce ejaculation. It should be noted that in using 30 per cent egg water, ejaculation occurred only with the fourth dose. In all the experiments conducted with various concentrations of egg water the contractions of the mantle preceded ejaculation. The latent period of ejaculation was somewhat lower than in the intact males.

TABLE XI

Sensory stimulation and ejaculation provoked by 0.8 per cent thyroidin solution. Temperature, 22–23° C. Observations of mantle reaction omitted.

Time	Latent Period in Seconds		
	Tentacle	Muscle	Ejaculation
2.26 P.M.	2.5	46.6	–
2.36	2.4	12.6	+*
2.40	12.6	—	–
2.54	20.6	—	61.2
2.50	1.8	—	11.2
3.40	—	38.2	12.0
3.45	—	—	11.4
3.50	—	—	8.6
4.06	—	—	12.2

* Positive reaction, latent period not determined.
— No reaction.

Tentacles were found to be very sensitive to 0.8 per cent of thyroidin, repeated application of which resulted in a complete narcosis of these organs. Paralysis of tentacles did not interfere with ejaculation (Table XI) which proceeded in a normal way. There is no evidence at present that the receptors of sexual stimuli are located in the tentacles and the observation that ejaculation may be provoked when the latter are paralyzed speaks against this assumption. The location of the receptors still remains to be found. Experiments with egg water suggest that they may be present in the mantle.

Biological Significance of Spawning Reactions of O. virginica

It has been shown above that in both sexes spawning can be induced by raising the temperature, by chemical stimulation, or by the combined action of both stimuli. This dual control is of importance, since a great percentage of physiologically ripe adult oysters as a rule do not respond to thermic stimulation alone. As one can see from Table XII, only about 50 per cent of males and a considerably smaller proportion of females respond to the increase in temperature. Spawning in the rest of them can be provoked by applying proper chemical stimulus. It was further proved that with regard to chemical stimulation of spawning, the females are specific in the sense that ovulation can be induced by no other chemical agent but the sperm of the oyster. The males, however, are non-specific, responding to foreign eggs and to a great variety of chemical compounds. The existence of a double control of spawning is obviously advantageous for the propagation of the species, for in case the temperature fails to reach a sufficiently high degree, chemical agents provide the necessary stimulus. Furthermore, chemical stimulation is instrumental in provoking more or less simultaneous spawning in a large oyster population; for as soon as ejaculation or ovulation begins in some of the individuals, ripe specimens next to them are stimulated to spawn and the process once started spreads rather rapidly over the entire community. Actual field observations made by the author in shallow water and in tanks in which ripe oysters were kept corroborate this statement. By recording the time and sequence of spawning in individual oysters, it was possible to see that ejaculation provoked in one of the males, placed in a corner of the tank, induced spawning of the females near-by, which in turn stimulated other males and in a very short time all the specimens in the tank were discharging their sex products. Artificial stimulation of spawning based on these findings was applied in the field

TABLE XII

Percentage of male and female oysters in which spawning was induced by temperature and chemical stimulation

Stimulus	Males				Females			
	1937		1938		1937		1938	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Rise of temperature	77	59.7	48	42.1	17	39.5	18	26.5
Chemical stimulus + temperature	45	34.9	40	35.1	24	55.8	30	44.1
Failures to respond	7	5.4	26	22.8	2	4.7	20	29.4
Total	129		114		43		68	

by Elsey (1936), who succeeded in inducing spawning of a large number of *O. gigas* in their natural environment on the bottom of Puget Sound.

Since discharged eggs, being heavier than water, sink to the bottom and after 24 hours become unfertilizable (unpublished observations of the author), the success of propagation of *O. virginica* is to a great extent determined by the simultaneity of spawning. Only under these conditions a large percentage of eggs will be fertilized, for proper timing of spawning in both sexes, accomplished through mutual stimulation, greatly increases the chance of insemination.

Because the males used in the laboratory experiments were found to be more responsive to thermic stimulation than the females, it was expected that under natural conditions spawning is initiated by the males. This expectation was confirmed by observation on ripe specimens kept in large outdoor tanks where in each instance the males were first to spawn.

The specificity of the female response to sexual stimulus appears to be useful for the propagation of the species because in the majority of cases the females discharge their eggs only when sperm of the same species is present in the water. The non-specific response of the male to a great variety of chemical stimuli is, however, instrumental in initiating spawning in the oyster population when temperature stimulus is not effective.

Experimental studies presented in this and previous papers of the author (Galtsoff, 1938*a, b*) indicate the necessity of revising the old concept of so-called critical temperature for spawning. According to the old views, summarized by Nelson (1928*a, b*) 20° C. constituted a critical temperature which exerted trigger-like effect on ripe oysters. Since this conclusion was based on field observations, no attempts were made to discriminate between the male and female spawnings. Present experimental studies show that there exist several "critical" temperatures for spawning of males and females, apparently determined by their physiological conditions. Furthermore, they indicate that in case of a combined action of temperature and chemical agent the effective temperature is always lower than in case of spawning induced by temperature alone. The idea of a single critical temperature effective in inducing spawning in all the oysters of a given population is not corroborated by observations and should be abandoned.

Chemical stimulation of spawning is not restricted to the genus *Ostrea*. It has been described for *Nereis* (Lillie and Just, 1913; Just, 1914), *Strongylocentrotus* (Fox, 1924), *Chiton* (Heath, 1905) and probably will be found in other invertebrates. In the marine forms deprived of external sex organs, it possibly constitutes the principal method of sexual excitation. As has been shown in the first part of this investigation, there is no significant difference in the anatomical structures of the gonads of the two sexes of oyster. The female has developed, however, a complex physiological coördination in the behavior of the adductor muscle, mantle, and ciliated epithelium which causes the eggs to pass through the gills into the pallial cavity and provides a very efficient mechanism for scattering them in the surrounding water. This greatly increases their chances for fertilization and survival. Observations on oysters operated on in such a way as to prevent the discharge of eggs through the gills (Galtsoff, 1938*a*) show that the majority of eggs ejected through the cloaca settle over a small area of the bottom and perish. The female spawning reaction can be considered therefore as a special physiological adaptation of a sedentary organism advantageous for the survival of the species.

On the other hand, the sexual reaction of the male is more simple than that of the female. One cannot help but correlate this with the fact that spermatozoa being very light are easily carried away by the current of water and are widely distributed in the sea. A special mechanism for their distribution would, therefore, be unnecessary.

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STUDIES ON THE LIFE HISTORY OF SPELOTREMA NICOLLI (TREMATODA: MICROPHALLIDAE) WITH THE DESCRIPTION OF A NEW MICROPHALLID CERCARIA

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Introduction and Historical Review

Although numerous investigators have observed and correlated certain stages in the life histories of species of *Spelotrema* and related genera of digenetic trematodes, no study has been reported in which the cycle has been completed and proved experimentally. During the summer of 1938, the writers fed metacercariae from the blue crab, *Callinectes sapidus,* to young herring gulls and obtained mature adult trematodes which proved to be a new species, *Spelotrema nicolli* Cable and Hunninen, 1938. At that time, the cercaria could not be found, but during the summer of 1939, the study has been completed and the life history of *S. nicolli* is now well understood.

In 1865, M'Intosh observed encysted microphallid larvae in the tissues of the green crab, *Carcinus maenas.* Lebour (1911) regarded this larva as the metacercaria of *Spelotrema excellens* Nicoll, 1907, and made the significant observation that a stylet was present and the ventral sucker was undeveloped in young metacercariae. Furthermore, she observed that the small monostome xiphidiocercaria, *C. ubiquita* Lebour, 1907, penetrated and encysted in crabs, and postulated that it was the cercaria of *S. excellens.* Brandes (1888) suggested that the metacercaria described by M'Intosh was the larva of *S. claviforme* (Brandes) while Nicoll (1907) maintained that there was a closer agreement between *S. claviforme* and the metacercaria which Lebour (1905) described and later (1911) named *C. littorinae-rudis.* Other microphallid metacercariae that have been found in crustaceans include a form which Villot (1879) found encysted in *Anthura gracilis* and postulated to be the larva of *Levinseniella brachysomum* (Creplin); *C. carcini* Lebour, 1908, from *Corophium grossipes* and *Gammarus duebeni; C. balani* Lebour, 1908, from *Balanus balanoides; C. ligiae* Lebour, 1914, from *Ligia oceanica;* metacercariae of *Levinseniella squatarolae* and *Spelophal-*

lus primas, described by Yamaguti (1934) from *Macrophthalmus dilatatus;* and unnamed *Spelotrema* metacercariae described by Stunkard (1932) from *Porcellanus longicornis* and *C. maenas.*

The observations of Lebour (1911) indicated that cercariae of the *Ubiquita* type may be larval microphallids. Other cercariae of this type include *C. indica LII* and *C. indica LXI* described by Sewell (1922) from *Amnicola travancorica* and *Digoniostoma cerameponia* respectively; *C. ubiquitoides* Stunkard, 1932, from *Littorina rudis* and *L. littorea;* a similar if not identical form described by Rees (1936) from the same hosts and also *L. obtusata;* and *C. nassicola* Cable and Hunninen, 1938, which occurs in *Nassa obsoleta* and is described more completely in the present paper.

Apparently, certain microphallid cercariae do not leave the molluscan host but lose their tails and encyst in the sporocyst. This behavior seems to be characteristic of *C. crispata* Pelseneer, 1906, in *Natica alderi; C. oocysta* and *C. pirum* found in *Paludestrina stagnalis* by Lebour (1907); *C. sinuosa* Sinitsin, 1911, from *Rissoa venusta; C. dimorpha* Sinitsin, 1911, from *Cerithiolum exille;* and *C. A.* Rothschild, 1936, from *Peringia ulvae.*

Experimental studies have demonstrated portions of the life histories of certain microphallid trematodes. Rothschild (1937) gave an adequate discussion of these studies and reported that she obtained a species of *Maritrema* when encysted *C. oocysta* were fed to gulls. More recently, Sheldon (1938) has found that metacercariae occurring in crayfishes develop into adults of *Maritrema medium* when fed to laboratory mice. Young (1938) has obtained stages in the life cycle of *Levinseniella cruzi* (?) Travassos, the cercaria of which occurs in *Olivella biplicata* and encysts in the sand crab, *Emerita analoga.* Young did not feed metacercariae to birds but concluded from morphological comparisons that the adult was a species occurring in shore birds.

The potential danger of species of *Spelotrema* as human parasites has been demonstrated by Africa and Garcia (1935), who found *Heterophyes brevicaeca* (renamed *Spelotrema brevicaeca* by Tubangui and Africa, 1938) associated with acute cardiac dilatation and discovered the eggs of this parasite in the heart tissue.

Materials and Methods

Throughout the present investigation, living material has been studied as much as possible since it was found to be far more favorable than fixed and stained specimens for most observations. Since all crabs contained metacercariae from natural infections, it was necessary to isolate those for experimental purposes for a time sufficient to permit encystment and growth of any recently acquired parasites that might have been present. In this manner, experimental infections could be detected by finding migrating larvae or very small metacercariae.

In studying the larger metacercariae, considerable difficulty was experienced in removing the larvae from the cysts without injury. This was done most successfully with the aid of a small glass tube drawn out and cut off with scissors at a point where the diameter was slightly smaller than that of the cyst. With aid of forceps, it was possible to rupture the cyst by forcing the capillary glass tube over it. Birds were infected by feeding them several hundred metacercariae placed in the body cavity of small fishes. Development of the eggs was followed by teasing adult worms and keeping the eggs thus obtained for study in dishes of sea water which was changed twice daily.

Conventional methods were employed in studying the various stages, neutral red and Nile blue sulphate being used for *intravitam* staining, paracarmine for whole mounts, and Delafield's haematoxylin with eosin counterstain for sectioned material. Unstained metacercariae in the crab's tissues were dehydrated and mounted in damar and also passed gradually into glycerine and mounted in glycerine jelly. All drawings except those indicated as diagrammatic were made with the aid of projection equipment, some of the details being added free-hand. All measurements given below are in millimeters and are from living material except in the case of adults which were stained whole mounts.

Observations

The Experimental Proof of the Life History

It has been known for several years that practically every blue crab in the Woods Hole area is infected with a microphallid metacercaria. Before attempting to obtain the cercarial and adult stages, a careful study of natural infections was made to find out as much as possible concerning the nature of the cercaria and the adult. Since the older metacercaria was well differentiated, it could be identified positively as a species of *Spelotrema*. During the summer of 1938, each of three young herring gulls was given an initial feeding of metacercariae, a second feeding 15 days later, and killed after three days. All three birds yielded large numbers of adult worms which could be separated readily into two size groups corresponding to the ages of the infections. Three control birds were negative for the same species but contained other parasites as did the experimental animals. In examining the birds, the cloaca and large intestine were severed just above the ceca and the small intestine divided into approximately four-inch lengths which were examined separately.

Most of the worms occurred in the two segments of the small intestine above the terminal one although a few were recovered both above and below this level. They were never found in the ceca. During the summer of 1939, three additional birds were infected experimentally, one being fed metacercariae twice, 17 and 3 days before it was killed and examined, another twice, 25 days and 12 hours, while the third was killed 36 hours after a single feeding of cysts. Since all of these birds became heavily infected and three additional controls were negative, it is concluded that the worms recovered were adults of the metacercariae fed. This conclusion is supported by the close agreement between the older metacercaria (Fig. 7) and the adult (Fig. 10).

A careful study of young metacercariae gave information of considerable significance in the search for the cercaria. It was determined that the cercaria would be of the Ubiquita type since a large stylet was present and the ventral sucker was undeveloped, From very young metacercariae, the exact size and shape of the stylet were determined.

During the summer of 1938, we were able to find only one cercaria of the Ubiquita type and this occurred in *Nassa obsoleta* collected at Sippewissett. Three small blue crabs were exposed to large numbers of these cercariae with negative results. This was expected since there were considerable differences in the shape and size of the stylets of young metacercariae and the cercariae from *N. obsoleta*. An examination of several thousand *N. obsoleta,* collected at Waquoit Bay where infected crabs were abundant, did not yield a single infection with the species found at Sippewissett. In view of these results, it was concluded that the cercaria of *S. nicolli* remained to be found. Early in the summer of 1939, the study was resumed from an ecological viewpoint. Due to the migratory habits of the blue crab, it was necessary to determine first whether infection occurred in open water or after migration into bays and inlets. To answer this question, crabs were collected from Waquoit Bay at Menauhant which is at least four miles by water from the only entrance to the bay. Since these crabs contained very young metacercariae only 0.05 mm. in diameter, it seemed probable that the infections were acquired in the bay. Then followed a systematic collection and examination of all species of mollusks that could be found where infected crabs occurred. Large numbers of *Nassa obsoleta, N. vibex, Littorina littorea, L. rudis, Melampus bidentatus, Mitrella lunata, Crepidula fornicata, Venus mercenaria, Modiolus modiolus,* and *Pecten irradians* were examined and found negative for ubiquitous cercariae. Finally, a larva of the type sought was found in the very small snail, *Bittium alternatum,* which occurred in large numbers on seaweed growing in one to four feet of water. The cercaria seemed to be identical with the form in the blue crab, especially in respect to the size and shape of the stylet. To test this apparent relationship, a small crab that had been isolated for three weeks was exposed to the cercariae almost continuously for seven days before it was killed. Upon examination, certain fibers were found to contain not only a few older cysts from natural infections but also numerous very young metacercariae (Fig. 19) most of which had not encysted. The young encysted larvae were undoubtedly from the experimental infection and identical with those recovered repeatedly from naturally infected crabs that had been collected only a short time before examination. Two additional crabs were exposed to cercariae and both became heavily infected.

The manner in which the cercariae entered the crab remained to be determined. The larva is not a powerful swimmer and when disturbed it usually ceases swimming for a few seconds. This behavior suggested that when the larvae come near the crab and are disturbed by its respiratory activity, they are carried passively into the gill chamber. With this possibility in mind, a small crab was exposed for 45 minutes to a large number of cercariae and then killed and examined. Two active larvae were found in the heart and a number was recovered from the efferent branchial vein of each gill. Since no larvae were recovered from the afferent vessels, it seems that they either penetrated the gill lamellae and passed by way of the outer lamellar sinuses to the efferent vessels, or bored directly into the efferent veins. The fact that no larvae were observed in the lamellae indicates that the cercariae normally lodge in the interlamellar spaces, which afford excellent protection, and make their way to the bases of the lamellae where they bore directly into the efferent veins (Fig. 18).

Description of Stages in the Life History of Spelotrema nicolli

Adult (Figs. 9–12).

Specific Diagnosis.—Microphallidae with characters of the genus *Spelotrema.* Total length 0.51–.58 (average 0.54); maximum width of forebody 0.21–.27 (0.24); width at mid-body 0.21–.25 (0.23); width hind-body 0.32–.37 (0.34); width oral sucker 0.05–.06 (0.056), ventral sucker 0.05–.065 (0.058). Distance from center of ventral sucker to posterior end of body 0.2–.24 (0.22). Maximum length prepharynx 0.035; esophagus length 0.12–.2 (0.16); ceca average 0.14 long, divergent, and not reaching posterior margin of ventral sucker. Diameter of penis 0.019–.024 (0.021); seminal vesicle 0.07–.09 (0.085) by 0.015–.045 (0.035). Testes about equal in size, 0.1–.13 (0.11) by 0.05–.075 (0.06). Ovary right, anterior and adjacent to testis of that side, 0.08–.09 (0.085) by an average of 0.05. Laurer's Canal present, shell gland dorsal to oötype; uterus voluminous, extending anterior to testes as far as ceca. Vitellaria diffuse, not observed as distinct lobes. Eggs very numerous, 0.018–.022 long by 0.009–.011 wide.

Host (experimental). *Larus argentatus,* localizing near posterior end of small intestine.

Locality. Woods Hole, Massachusetts, U. S. A.

Type specimens. Holotype No. 9232, Helminthological Collection, U. S. National Museum; paratypes in authors' collections.

The forebody of *S. nicolli* is armed with prominent scale-like spines arranged in imbricated rows. These spines decrease in number and size at the level of the acetabulum but may be found by careful examination to be scattered in small numbers over the hind-body, almost to the posterior end. Prominent refractile glands are scattered beneath the cuticula of the forebody and are so numerous that other structures in that part of the body are obscured in living specimens. A cluster of similar glands have ducts which converge at the margin of the genital pore (Fig. 11) and might be mistaken for the deeper prostate glands. The cytoplasm of the subcuticular glands is homogeneous in appearance. The ceca have thick walls of gland-like cells and are surrounded by prominent masses of granular cells having the appearance of glands.

The excretory system of the adult is the same as described below for the older metacercaria. The reproductive system as a whole may be observed much easier in living worms than in either whole mounts or sectioned material. Since the presence of numerous eggs in the uterus obscured many details of the reproductive system, some of the feeding experiments were devised primarily to provide young worms in which only a few eggs were present. The details of structure and the functioning of various generative organs were observed very favorably when such worms were mounted dorsal side up under as little coverglass pressure as possible and studied with the oil immersion objective.

The penis (Figs. 9–12) is a pyriform, muscular organ, situated in the genital atrium at the left of the ventral sucker. It is penetrated by the ejaculatory duct which seems to receive the openings of a number of elongate prostate glands just before it enters the penis. The ejaculatory duct is moderately sinuous. It expands to form the seminal vesicle, there being no indication of a double-walled vesicle. On the right, the vesicle narrows to form the short vas deferens which extends posteriorly, dorsal to the ventral sucker, and divides to form the delicate vasa efferentia. From this Y-shaped division, the vasa efferentia pass posterolaterally, very close to the dorsal side of the body, and connect with the testes.

The ovary is frequently somewhat triangular in shape. From the median edge, the oviduct extends posteriorly as a thick-walled, sinuous tube. Close to the median edge of the right testis, the oviduct enters a prominent, spherical bulb which has thick, muscular walls. Because of its function as described below, this bulb is called the ovijector. A small internal papilla marks the point at which the oviduct enters the ovijector, and a more prominent papilla at the opposite side of the bulb surrounds the entrance to the oötype (Fig. 12). Proceeding from the ovijector, the oötype turns abruptly ventrad and is joined immediately by the Laurer's Canal which extends dorsally in a sinuous path and opens near the median line. From the ovijector to the opening of Laurer's Canal, the wall of the oötype is composed of overlapping cells. Beyond the opening, the wall is thinner. The oötype extends posteriorly for a short distance and then bends dorsally, extending anteriorly not far beneath the dorsal surface of the body. It is ciliated almost to the point where the dorsal loop receives the opening of the vitelline reservoir. Just anterior to this opening, the oötype receives the numerous slender ducts of the shell gland, all of which lies above the oötype. The vitellaria are composed of diffuse masses situated below and behind the testes. The vitelline ducts extend forward below the testes and unite in the median line to form the vitelline reservoir which passes backward and upward to join the oötype.

When living worms are studied as has been described, the process of egg formation can be observed in considerable detail. The oöcyte (assuming that meiotic divisions do not occur until sperm penetration, as is true of certain other trematodes) gradually separates from the ovarian mass while the preceding egg is formed. It is started and propelled down the oviduct, however, by a constriction passing along the oviduct. The oöcyte moves slowly at first and then with increasing rapidity. It remains for only an instant in the ovijector which contracts immediately, expelling the oöcyte forcibly into the oötype. This contraction of the ovijector is followed by a localized enlargement of the oötype in which currents produced by cilia rotate the oöcyte rapidly for a few moments. Then the oöcyte is carried along the oötype by a series of contractions. Since a few sperms are usually present in the ovijector, fertilization (i.e., sperm penetration) could occur there. Sperms were seen attempting to pass up the oviduct as the oöcyte descended but have never been observed in the ovary or oviduct during its inactive phase. The passage between the ovijector and the oviduct remains closed most of the time. Since the ovijector does not always contain sperms and the oöcyte passes through it almost too rapidly to be observed, it seems probable that fertilization occurs normally in the oötype which is always crowded with sperms. The rapid and fairly prolonged rotation of the oöcyte in the oötype would facilitate fertilization by aiding the sperm to engage and coil about the oöcyte.

The zygote pauses at the opening of the vitelline reservoir which then begins to contract. These movements become more and more forcible until several masses of vitelline material are expelled into the oötype. The zygote, surrounded by this material, then passes just beyond the openings of the shell gland into the more muscular part of the oötype which may be termed the egg chamber. Excess vitelline masses and occasional sperms are swept by the cilia of the oötype back to the Laurer's Canal through which they escape from the body. Before the shell is formed, vigorous contractions of the egg chamber break up the masses of vitelline material into fine granules. The egg is kept in constant motion by contractions of the oötype and may be turned end for end several times before the shell is formed completely. A much stronger contraction begins back of the completed egg and ejects it into the uterus. Under observation, several attempts may be necessary to expel the egg, due perhaps to the interference of coverglass pressure with normal function. At the moment the egg passes into the uterus another oöcyte begins to move down the oviduct and the process is repeated. In one instance, it was noted that at room temperature 24 minutes elapsed between the descent of one oöcyte and that of the succeeding one. At the body temperature of the host, however, egg formation must proceed much more rapidly than observed to account for the number of eggs present in young worms.

The eggs are colorless when first formed but become yellow with age. Small, abnormally shaped eggs (Fig. 13) with terminal knobs are often seen in the uterus and such eggs are produced frequently after removal of the worms from the host. Occasionally, a specimen is observed in which practically all the eggs are of this type. Abnormal eggs almost always contain only vitelline material and are formed apparently when oöcytes fail to descend.

The more significant measurements of described species of *Spelotrema* are given in Table I. As Odhner (1905) pointed out, the size of the penis is remarkably constant for a given species and this character alone distinguishes *S. nicolli* from all other species of *Spelotrema* except *S. pygmaeum* (Levinsen). However, *S. nicolli* and *S. pygmaeum* differ significantly in respect to body size and shape, sucker ratios, extent of the uterus, and nature of the vitellaria. Both Odhner and Nicoll (1909) gave 0.5 mm. as the maximum length of *S. pygmaeum;* practically every stained specimen of *S. nicolli* exceeds this length and the average for moderately contracted living specimens is over 0.6 mm. Concerning the body shape of *S. pygmaeum,* Odhner stated that, at all degrees of contraction, the width of the body increased without interruption from the anterior towards the broadly rounded posterior end. He observed this body form in all of his material and considered it a specific character in differentiating *S. pygmaeum* from *S. simile* (Jägerskiöld, 1900). Except when extended greatly, the body of *S. nicolli* is constricted at the level of the ventral sucker. This characteristic is noticed particularly in living specimens and, although present in stained material, may be rendered less prominent by pressure when specimens are fixed under the coverglass. According to Odhner, the oral sucker of *S. pygmaeum* is usually larger than the ventral sucker, although exceptions were noted. The opposite is true of *S. nicolli;* the ventral sucker is nearer the middle of the body than in *S. pygmaeum.* In *S. nicolli,* the hind-body is larger and more expanded than in *S. pygmaeum.* In Odhner's figure, the uterus of *S. pygmaeum* does not extend anterior to the testes and this condition may be regarded as typical since he had an abundance of material. In *S. nicolli,* the uterus always extends anterior to the testes; this condition is characteristic even of the empty uterine coils of immature worms, especially on the antovarian side, and does not depend

TABLE I

Comparison of species of Spelotrema

Species	*S. pygmaeum*	*S. claviforme*	*S. simile*	*S. excellens*	*S. brevicaeca*	*S. nicolli*
	(Levinsen)	(Brandes)	(Jägerskiöld)	Nicoll	(Africa and Garcia)	Cable and Hunninen
Citation	Odhner (1905)	*Nicoll (1907)	Odhner (1905)	*Nicoll (1907)	Tubangui and Africa (1938)	Present Report
Length (mm.)	Not exceeding 0.5	0.23–.4	0.45–.6	0.71–1.39 (av. 0.91)	0.5–.7	0.51–.58 (av. 0.54)
Breadth (maximum) (mm.)	0.2–.3	0.17		0.37–.49 (av. 0.41)	0.3–.4	0.32–.37 (av. 0.34)
Oral sucker diameter (mm.)	0.04–.053	0.038	0.046–.058	0.068–.086 (av. 0.076)	0.065–.095	0.05–.06 (av. 0.056)
Ventral sucker diameter (mm.)	0.037–.048	0.031	0.049–.062	0.062–.081 (av. 0.071)	0.08–.105	0.05–.065 (av. 0.058)
Male papilla diameter (mm.)	0.021–.023	0.013–.014	0.04	0.05–.065	0.03–.045	0.019–.024 (av. 0.021)
Eggs	0.02–.023 by 0.012	0.02–.024 by 0.011–.014	0.023–.026	0.023–.025 by 0.01–.013	0.015–.016 by 0.0094–.01	0.018–.022 by 0.009–.011

* An obvious misplacement of the decimal point in several of Nicoll's measurements has been corrected.

on the degree of maturity. Odhner's figure also shows approximately 100 eggs in the uterus of *S. pygmaeum*. This number is exceeded in specimens of *S. nicolli* that have been in the bird only 36 hours and the uterus of older worms contains several hundred eggs. Odhner also described eight distinct vitelline masses on each side of *S. pygmaeum* and represented them as a cluster somewhat posterior to the testes. In *S. nicolli,* the vitellaria are more diffuse and overlap the posterior edge of the testes. In some specimens, vitelline lobes have been observed but they are fused in such a manner that a count can not be made with certainty. A further difference between *S. nicolli* and *S. pygmaeum* is indicated by Nicoll's (1909) statement that the ceca of *S. pygmaeum* reach the posterior margin of the ventral sucker; the ceca of *S. nicolli* rarely extend posterior to the middle of the sucker.

The metacercaria (Figs. 4–8).

The metacercariae occur only in certain slender fibers which extend from the viscera of the crab to the bases of the legs. These fibers are very tough and elastic and seem to be composed of connective tissue since they are not striated and separate readily from the groups of striated muscles at the bases of the legs. In cases of heavy infection, the fibers are greatly enlarged and filled with metacercariae.

Metacercariae were observed ranging from 0.05 to almost 0.5 mm. in cyst diameter. The stylet is retained until the metacercaria attains considerable size. It is gradually absorbed, however, and never shed or broken off as in the case of some xiphidiocercariae. The young metacercaria secretes a very thin primary cyst wall (Fig. 5*a*) which is elastic and changes shape with the movements of the worm. At a later stage, the cyst becomes enclosed in a mass of granular tissue which is of host origin and contributes to the formation of secondary cyst layers. In the older metacercaria (Fig. 8), the cyst membranes are of two types, an outer radially striated layer which is very thick and exceedingly tough, and one to three hyaline inner layers which become fairly thick. The entire cyst may be embedded in a mass of fibrous tissue of host origin.

When removed from the cyst, the older metacercaria is practically as large as the adult which it resembles closely except that eggs are absent. After excystation, egg production must begin in a short time since the young adults contain eggs after only 12 hours in the avian host.

The excretory formula of the metacercaria (Fig. 6) is $2[(2+2)+(2+2)]$; it remains unchanged in the adult stage.

Cercaria (Figs. 16, 17).

Specific Diagnosis.—Small " monostome " xiphidiocercaria with the characters of the Ubiquita Group of Sewell (1922). Body contracted less than 0.1 long, extended 0.24, average 0.11; covered with small scale-like spines in imbricated rows. Tail 0.04–.1 long, with fine cuticular annulations. Oral sucker 0.026 long, provided with stylet 0.016 long. Remainder of digestive system not observed. Four pairs of cephalic glands of two types, a larger anterior pair on each side with ducts extending forward together and then separating, the median duct crossing the oral sucker and joining the lateral duct at the side of the sucker where both turn ventrad, opening on ventral surface of body near anterior end; a smaller posterior pair with ducts passing anteriorly together with lateral duct of anterior gland and opening near tip of stylet. Excretory vesicle U-shaped, each arm receiving a main collecting tubule which extends forward half way to cephalic glands, there dividing to form anterior and posterior secondary tubules, each of which receives capillaries of two flame cells. Excretory formula: $2[(1+1)+(1+1)]$. Develop in oval or elongate sporocysts with or without terminal knobs.

Host: *Bittium alternatum* (Say).

Locality: Woods Hole, Massachusetts, U. S. A.

The cercaria of *S. nicolli* resembles other larvae of the Ubiquita type but differs from all described species in the nature of its penetration glands, size and shape of stylet, and molluscan host. The stylet is symmetrical in dorsal aspect but asymmetrical when viewed from the side. The barb is quite hard and persists whereas the shaft is fragile and frequently disintegrates when living cercariae are mounted under consider-

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EXPLANATION OF PLATE I

(FIGS. 1–3, *Cercaria nassicola*)

FIG. 1. Cercaria in dorsal aspect.

FIG. 2. Stylet, ventral aspect.

FIG. 3. Anterior end of cercaria in side view, showing arrangement of cephalic gland ducts.

(FIGS. 4–6, *Spelotrema nicolli*)

FIG. 4. Older metacercariae in tissue of crab.

FIG. 5. *A*. Younger metacercaria in which stylet is still present. *B*. Older metacercaria.

FIG. 6. Excretory system of metacercaria.

ABBREVIATIONS

AO, anterior openings of posterior cephalic glands.
DG_1, anterior cephalic gland duct.
DG_2, posterior cephalic gland duct.
DO, dorsal opening of anterior cephalic gland duct.
G_1, anterior cephalic glands.
G_2, posterior cephalic glands.
S, stylet.
VO, ventral opening of anterior cephalic gland duct.

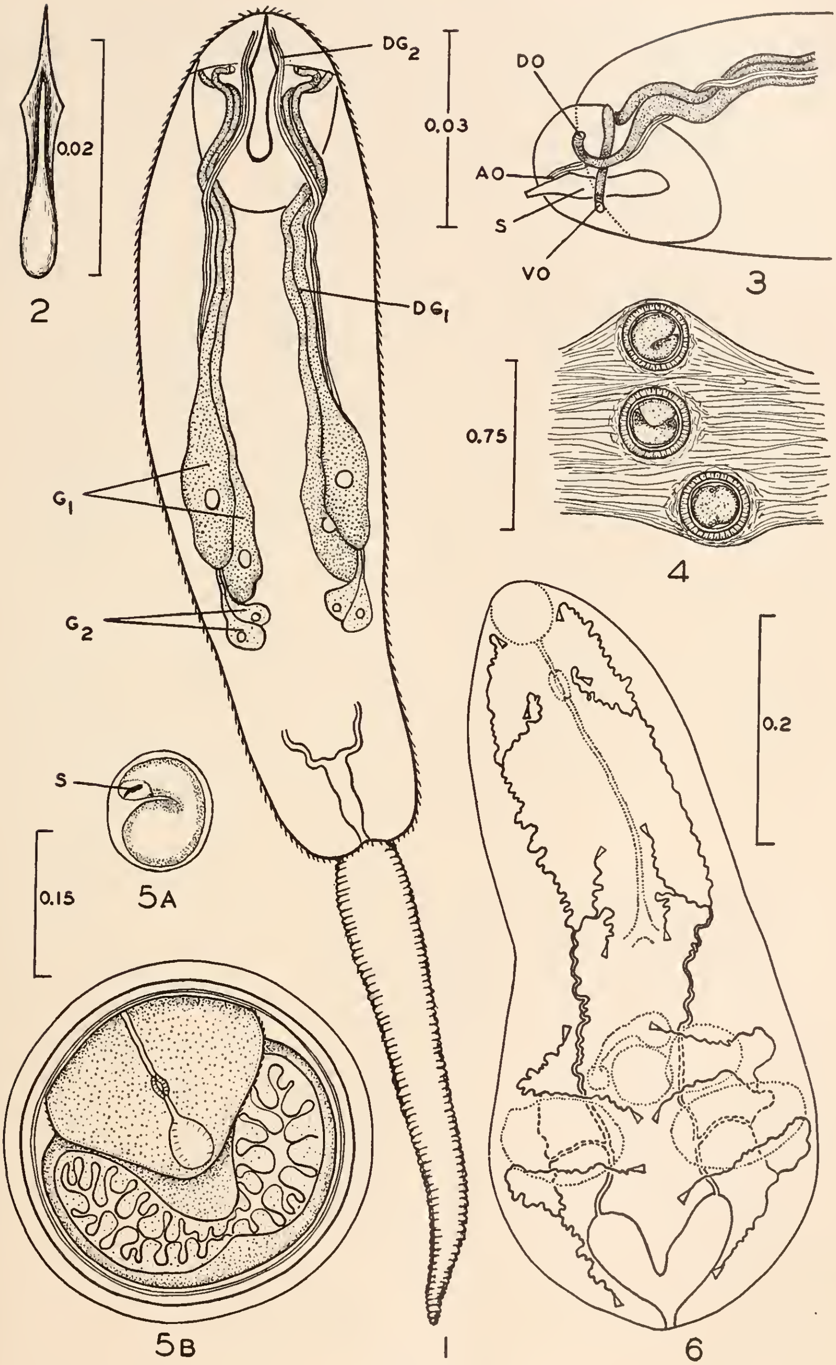
0.02
2
DG2
0.03
DG1
G1
G2
DO
AO
S
VO
3
0.75
4
0.2
S
0.15
5A
5B
1
6

Plate I

able coverglass pressure. On each side, the anterior pair of cephalic glands stains intensely with neutral red and Nile blue sulphate. Their contents move readily into the ducts which consequently become very conspicuous. The two posterior glands do not stain appreciably with *intra-vitam* stains and their ducts are extremely difficult to observe in cercariae emerging spontaneously from the snail. However, the cercariae encyst readily on the slide and then the ducts of the posterior glands become distinct. Before such encystment, the contents of the anterior glands are thrown out and their ducts become invisible. The ducts of both types of glands have been observed simultaneously only a few times, usually in larvae obtained by crushing the snail. Encystment on the slide occurs very rapidly, especially when *intra-vitam* stains are used. Cystogenous material is secreted over the entire surface of the body, the tail is lost, and the cyst forms with the body more or less extended instead of flexed as in normal encystment in the crab. The larva sometimes ruptures the cyst and emerges but dies after a short time.

Upon emerging from the snail, the cercariae swim almost continuously with the posterior part of the body flexed ventrally and the tail lashing vigorously. During short rest periods, the body remains flexed for a moment and then usually extends and contracts once or twice before swimming is resumed. It has been mentioned already that swimming may cease in response to stimulation. To observe this behavior more closely, 10 cercariae were isolated in separate culture dishes and each was stimulated five times at 15-second intervals by dipping a needle

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Explanation of Plate II

(All figures concern *Spelotrema nicolli.*)

Fig. 7. Morphology of metacercaria removed from cyst.

Fig. 8. Details of cyst structure.

Fig. 9. Slightly diagonal cross-section of adult, showing details of the genitalia.

Fig. 10. Adult. Holotype in ventral aspect.

Abbreviations

E, esophagus.
ED, ejaculatory duct.
EV, excretory vesicle.
GA, genital atrium.
GO, opening of subcuticular gland.
GP, genital pore.
I, intestine.
MP, male papilla or penis.
OS, oral sucker.
OV, ovary.
PH, pharynx.
PP, prepharynx.
PR, prostate cells.
SV, seminal vesicle.
T, testis.
U, uterus.
V, vitellaria.
VA, vagina.
VD, vitelline duct.
VS, ventral sucker.

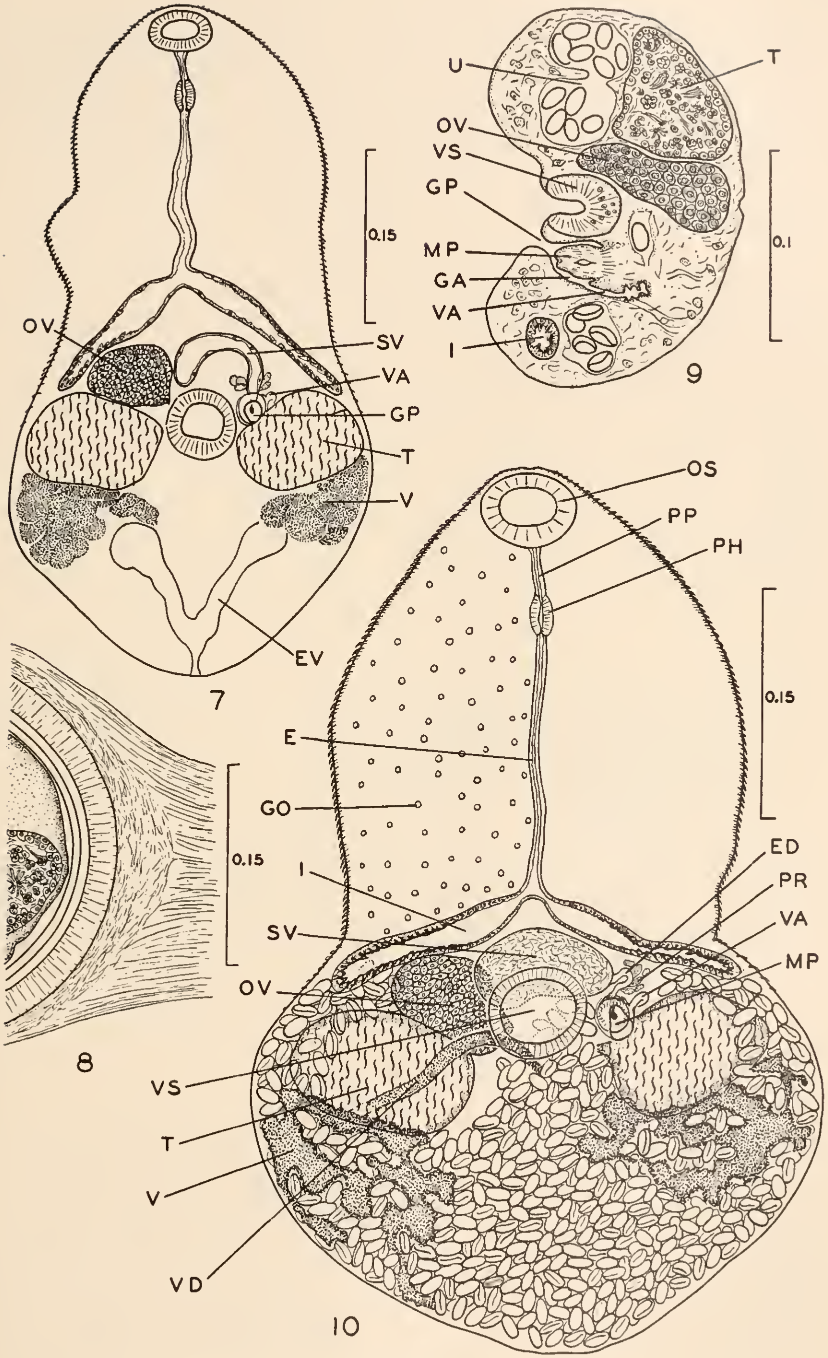
OV
SV
VA
GP
T
V
EV
0.15
7
U
T
OV
VS
GP
MP
GA
VA
I
0.1
9
0.15
8
OS
PP
PH
0.15
E
GO
I
SV
OV
VS
T
V
VD
ED
PR
VA
MP
10

Plate II

into the water. Four larvae responded to every stimulus with immediate cessation of swimming, three responded to four of the five stimulations, two to three, and one to only a single stimulus. Of a total of fifty observations, 39 gave positive responses, swimming movements ceasing in each case for 1.3 to 7.7 seconds, with an average of 2.9 seconds.

The sporocyst (Fig. 15).

There are probably two sporocyst generations of *S. nicolli* in *B. alternatum* but only that giving rise to cercariae has been observed. The smallest sporocyst observed measured 0.06 mm. long. The younger sporocysts are oval in shape and contain no mature cercariae, while the older ones are elongate and frequently constricted at one or both ends. The birth pore is terminal. It is rather difficult to separate some of the knobbed sporocysts from the host tissue and when they are removed, there is evidence in some cases that parts are broken off. The constricted sporocysts might be interpreted either as indicating fission or

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Explanation of Plate III

(All figures concern *Spelotrema nicolli.*)

Fig. 11. Adult. Free-hand sketch showing group of subcuticular glands with ducts converging at the genital pore.

Fig. 12. Reproductive system of adult (semi-diagrammatic).

Fig. 13. Normal and abnormal eggs.

Fig. 14. Embryonated egg containing miracidium.

Fig. 15. Sporocysts.

Fig. 16. Cercaria, dorsal view.

Fig. 17. Stylet of cercaria, (*A*) dorsal and (*B*) lateral views.

Fig. 18. Diagram indicating probable route of cercariae in penetrating the crab's gill.

Fig. 19. Portion of a fiber of crab's tissue containing young metacercariae from experimental infection.

Abbreviations

AF, afferent branchial vessel.
BP, birth pore.
DG_1, anterior cephalic gland duct.
DG_2, posterior cephalic gland duct.
ED, ejaculatory duct.
EF, efferent branchial vessel.
EV, excretory vesicle.
G_1, anterior cephalic glands.
G_2, posterior cephalic glands.
GL, gill lamella.
LC, Laurer's Canal.
MP, male papilla or penis.
OD, oviduct.
OJ, ovijector.
OO, oötype.
OS, oral sucker.
OV, ovary.
PR, prostate cells.
S, stylet.
SG, shell gland.
SV, seminal vesicle.
T, testis.
U, uterus.
VA, vagina.
VD, vitelline duct.
VR, vitelline reservoir.
VS, ventral sucker.

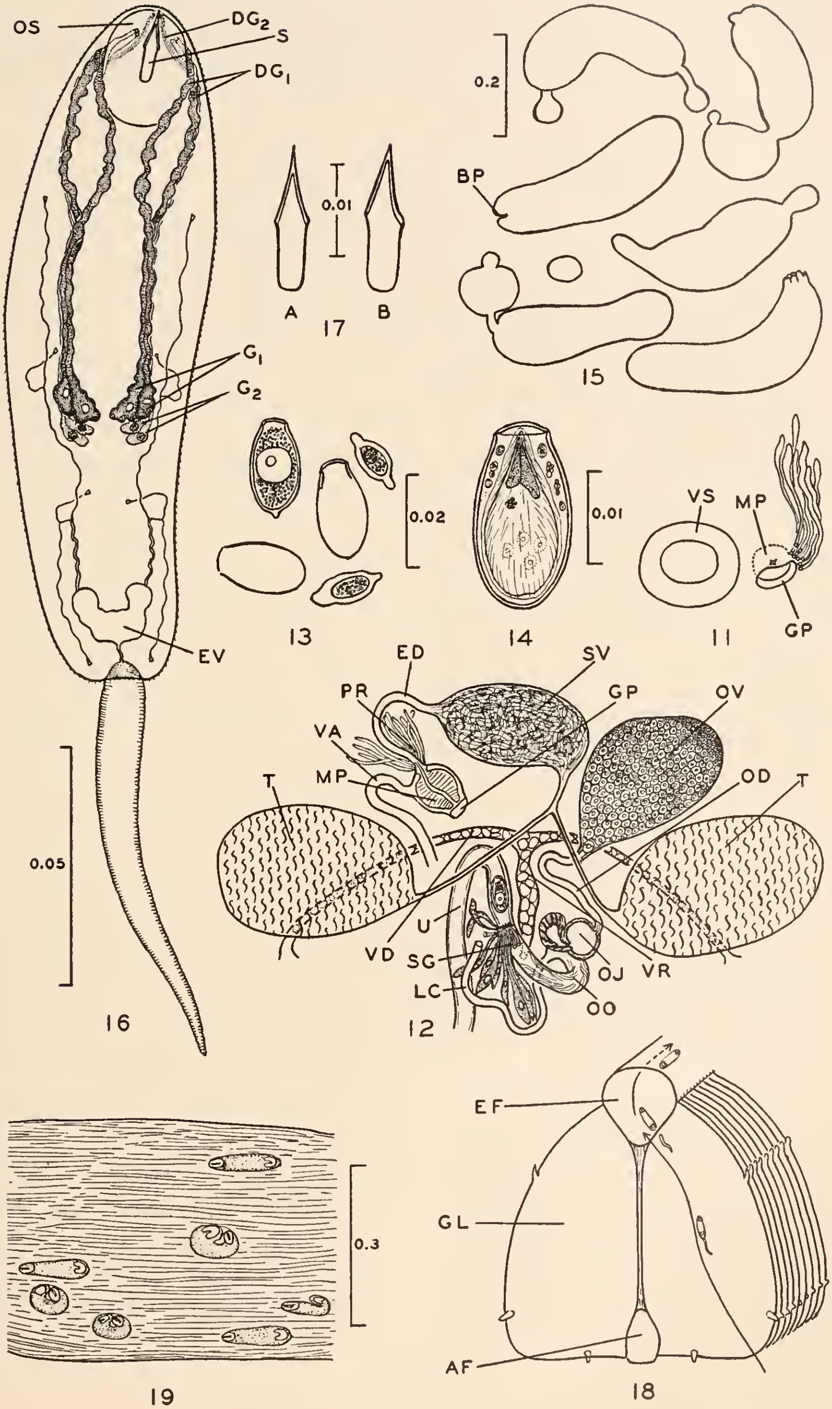
OS
DG2
S
DG1
G1
G2
EV
0.05
16
A
0.01
B
17
0.2
BP
15
0.02
13
0.01
14
VS
MP
GP
11
ED
SV
PR
GP
OV
VA
MP
OD
T
T
U
VD
SG
LC
OJ
VR
OO
12
EF
GL
AF
18
0.3
19

PLATE III

merely as older sporocysts, portions of which are exhausted. Other ubiquitous cercariae develop in oval sporocyts, a fact which affords an additional difference between the cercaria of *S. nicolli* and described species.

The egg and miracidium (Figs. 13, 14).

The process of egg formation has been described above. Development of the miracidium is exceedingly difficult to follow because of its small size. When gravid adult worms were teased, a portion of the eggs obtained developed in a normal manner when placed in sea water which was changed frequently to provide oxygen. Development observed in these eggs seemed to be extremely slow and although it was followed for 30 days before the study had to be terminated, the miracidia showed no signs of activity.

The first part of the micracidium to appear distinctly is the anterior end which is seen as a small, granular cone directed toward the operculum. As development proceeds, the remainder of the miracidium becomes more definite in outline. The granules filling the anterior end appear to be the contents of gland cells similar to those of other miracidia. Just posterior to this granular mass is a spherical cluster of granules which may be an eye-spot. Except for a few germ cells, the remainder of the miracidium is poorly defined. It is quite possible that the miracidia died before development was completed since movement was never observed.

Description of Cercaria nassicola Cable and Hunninen, 1938
(Figs. 1–3)

Specific Diagnosis—Small "monostome" xiphidiocercaria with the characters of the Ubiquita group. When moderately contracted, body length 0.1–.13, tail 0.07–.08. Entire body covered with prominent retrorse spines; tail finely annulated. Oral sucker 0.03 long; stylet 0.023 in length with tip truncate in lateral aspect. Two pairs of cephalic glands on each side, the anterior pair larger and with coarse granules, the posterior pair smaller and with finer granules. Larger glands stain intensely with neutral red, with one duct opening dorsolaterally, the other ventrolaterally in a groove encircling anterior end of body; smaller glands stain less intensely with neutral red, ducts open near tip of stylet. Develop in small oval sporocyts.

Host *Nassa obsoleta* (Say).
Locality: Woods Hole, Massachusetts, U. S. A.

C. nassicola is very delicate and disintegrates in a short time when subjected to any considerable coverglass pressure. For this reason, it has not been possible to determine the excretory formula. The vesicle and the beginning of the main collecting tubules were observed clearly only a few times. Decaudation occurs in a short time when the larvae are mounted for study but encystment has not been observed.

In a preliminary description of *C. nassicola,* it was stated that the tail is spinose. Further study has proved that this is not the case, but instead, the tail is very similar to that of the cercaria of *S. nicolli.* When the living cercariae are mounted for study, the cuticle of the tail becomes detached at the posterior margin of each annulation as disintegration begins and gives the tail a spinose appearance. This condition was encountered so consistently in earlier observations that it was misinterpreted.

The size and shape of the stylet and the arrangement of the cephalic gland ducts of *C. nassicola* are characteristic of the species. The ducts of the larger glands extend forward in the dorsal part of the body, a pair on either side. At about the middle of the oral sucker, the median duct of each pair loops upward, opening dorsolaterally in the groove. There is no marked separation of the ducts anteriorly as in the cercaria of *S. nicolli* and *C. ubiquitoides.* The ducts of the smaller posterior glands are very delicate. They extend anteriorly at the sides of the larger ducts which they cross at the posterior end of the oral sucker. From this point, they turn downward and forward around the oral sucker to open near the tip of the stylet.

Discussion

There is some confusion concerning the zoölogical status of the genera *Spelotrema* and *Levinseniella.* Tubangui and Africa (1938) expressed hesitation in referring *Heterophyes brevicaeca* to the genus *Spelotrema.* Young (1938) did not figure the adult stage of the species he referred to the genus *Levinseniella* and his incomplete description of this stage could have been that of a species of either *Levinseniella* or *Spelotrema.* As a matter of fact, the difference between these genera is quite evident but the manner in which the present conception of them has developed is confusing.

Stossich (1899) erected the genus *Levinsenia* to include all the microphallids known at that time, viz., *Distomum opacum* Ward, *D. brachysomum* Creplin, *D. pygmaeum* Levinsen and *D. macrophallos* von Linstow. In the same year, Lühe and Looss independently designated *L. brachysoma* as type species. Ward (1901) removed *L. opacum* to the new genus *Microphallus* and stated that Stiles and Hassall were to propose the name *Levinseniella* to replace *Levinsenia* (preoccupied) in a forthcoming paper which did not appear until 1902. Meanwhile, Jägerskiöld (1901), with full knowledge of Ward's statement concerning the intention of Stiles and Hassall, proposed the name *Spelotrema* for *Levinsenia* and designated *S. pygmaea* as type. If the law of priority should be applied to this case, *Spelotrema* Jägerskiöld, 1901, should be suppressed as a synonym of *Levinseniella* Stiles and Hassall in Ward, 1901, since Jägerskiöld stated subsequently (1904) " Spelotrema (= Levinseniella) " and therefore certainly regarded them as synonymous. His later (1907) conception of two distinct genera is valid, however, and must be accepted although he should not have retained for them names which he had regarded previously as synonyms. To suppress *Spelotrema* as a synonym of *Levinseniella,* and propose a new generic name for the species at present allocated to the genus *Spelotrema,* would probably increase rather than diminish the present confusion. For this reason, the writers are inclined to let the matter stand.

As regarded at present, members of the genus *Spelotrema* have a simple genital atrium containing a conical male papilla or penis, near the base of which the vagina enters the atrium. Species of *Levinseniella* have very different genitalia with complicated folding of the genital atrium. These generic differences are discussed fully by Jägerskiöld (1907).

Much emphasis has been placed in recent years on the value of information obtained from life history studies and excretory patterns in determining the relationships of digenetic trematodes. This information is gradually providing a natural system of classification based on fundamental relationships. As a result, it has been discovered that the taxonomic importance of certain morphological characters has been overemphasized in the past, particularly in the separation of familial and more inclusive groups. Studies on life histories and excretory systems have revealed in some instances hitherto unexpected relationships between morphologically dissimilar adult trematodes. On the other hand, it has been discovered that fundamentally dissimilar groups have been regarded as closely related by earlier workers who were misled by apparent morphological resemblances.

Perhaps no group of trematodes illustrates these facts more clearly than those that were included in the family Heterophyidae until only a few years ago. On the basis of morphological studies, Witenberg (1929) excluded certain genera from this family and proposed to unite those remaining with the opisthorchiids in the superfamily Opisthorchoidea. The life history studies of Stunkard (1930) on *Cryptocotyle lingua* and Vogel (1934) on *Opisthorchis felineus* gave such convincing evidence of the close affinities of the Heterophyidae and Opisthorchiidae that Vogel concurred in the opinion of Witenberg, renaming the superfamily Opisthorchioidea. Previously, Travassos and Viana had raised the heterophyid subfamily, Microphallinae Ward, to the status of a family, Microphallidae, to include *Microphallus* and related genera, all of which were among those Witenberg removed from the Heterophyidae. The observations of Lebour (1911) gave the first significant indications as to the nature of microphallid life histories. Subsequent studies afford indisputable evidence of the validity of placing *Spelotrema* and related genera in a distinct family. The validity of the name of this family, Microphallidae, depends on a more complete understanding of the morphology and life history of *Microphallus,* the type genus. However, the exact agreement between the excretory systems of *Microphallus, Spelotrema,* and *Maritrema* and other morphological similarities make it seem very likely that the retention of the name *Microphallidae* will be justified by further studies.

Rothschild (1937) and Stunkard (1938) suggested that the microphallids may be related to the Lecithodendriidae, a family which McMullen (1937) has placed in the superfamily Plagiorchoidea. On the basis of Carrère's (1936) study of the life history of *Maritrema rhodanicum,* McMullen places *Maritrema* in the Lecithodendriidae since Carrère reported that the cercaria of *M. rhodanicum* is of the Armatae type. In the writers' opinion, families of the Plagiorchoidea differ as significantly in respect to morphology and life history as do certain plagiorchoids and microphallids. It therefore seems reasonable to include the Microphallidae in the Plagiorchoidea.

The terminal portions of the reproductive system seem to be subject to extreme modifications as indicated by the differences observed in the microphallid genera, *Spelotrema, Maritrema, Levinseniella,* and *Microphallus.* The Heterophyidae (*sensu stricto*) also show considerable variation in the genitalia and genital suckers are by no means limited to this group. It is clear, then, that the apparent nature of the reproductive system, particularly the terminal portions, has been misleading in the separation of suprageneric groups, especially when careful studies of the genitalia have not been correlated with other characteristics.

SUMMARY

The life history of *Spelotrema nicolli* Cable and Hunninen, 1938, has been traced experimentally and the various stages have been described. The cercaria develops in sporocysts in the digestive gland of *Bittium alternatum* (Say). The blue crab, *Callinectes sapidus* Rathbun, serves as the second intermediate host. In experimental infections, the cercariae were found to penetrate the gills, and pass by way of the blood stream to the tissues. Metacercariae from naturally infected crabs were fed to young herring gulls, *Larus argentatus* Pontoppidan, from all of which numerous adult *S. nicolli* were recovered.

A re-description of *Cercaria nassicola* Cable and Hunninen, 1938, is given. This ubiquitous species occurs in the mud snail, *Nassa obsoleta* (Say).

The taxonomy and relationships of the Microphallidae are discussed.

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Vol. LXXVIII, No. 2 April, 1940

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

SPERM AGGLUTINATION IN THE KEYHOLE LIMPET, MEGATHURA CRENULATA

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F. R. Lillie (1913) demonstrated that the sea water in which ripe sea-urchin eggs have been standing acquires the property of causing an agglutination of the spermatozoa of the same species. This iso-agglutination of sperm by egg water has since been reported in a number of animals, principally in the echinoderms, as the following list shows—

Echinoderms:
  Arbacia punctulata (Lillie, 1913, 1914, 1919)
  Strongylocentrotus purpuratus (Loeb, 1914; Lillie, 1921)
  Strongylocentrotus franciscanus (Loeb, 1914; Lillie, 1921)
  Asterias forbesii (Glaser, 1914; questioned by Just, 1930)
  Echinarachnius parma (Just, 1915, 1919)
  Paracentrotus lividus (Just, 1929)
  Echinus microtuberculatus (Just, 1929)
  Echinus esculentus (Carter, 1932)
  Echinometra subangularis (Southwick, 1939)
Annelids:
  Nereis limbata (Lillie, 1913)
  Platynereis megalops (Just, 1915)
Mollusks:
  Katharina tunicata (Sampson, 1922)

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An interesting feature of the agglutination reaction, as described by Lillie, is that the agglutinates break up spontaneously. The clumps form very quickly after the addition of egg water, then after a few seconds or a few minutes, depending upon the concentration of egg water employed, reversal occurs. After complete agglutination and reversal the sperm cannot be re-agglutinated although they are alive and motile. The impression has been given, in the work on agglutination (see Lillie, 1919, p. 122; Lillie and Just, 1924, p. 489) that the spontaneous reversal is characteristic of iso-agglutination (i.e. by egg water of the same species), while non-reversal occurs only upon hetero-agglutination (i.e. by foreign egg water or other agents). In *Nereis,* however, Lillie himself has mentioned (1913, p. 552) that "the agglutinations are essentially permanent," and in *Katharina,* Sampson (1922) notes that some of the agglutinates reverse whereas others remain permanent.

In a recent note to *Science* it has been reported (Tyler and Fox, 1939) that the keyhole limpet, *Megathura crenulata,* exhibits an agglutination reaction which does not spontaneously reverse within the period during which the sperm remain viable. It has since been found that reversal may occur under certain conditions, particularly when excess sperm is employed. It was also reported that the form of the agglutinates is such as to indicate agglutination by the tails as well as by the heads of the spermatozoa, the picture being similar to that described by Sampson (1922) for *Katharina* sperm in egg water and by Henle, Henle and Chambers (1938) for bull sperm in anti-sera. In the present article a more detailed account of the agglutination reaction is presented together with some information concerning the source of the agglutinin and the specificity of the reaction. In a subsequent article, work on the chemical and physical properties and on the preparation of active concentrates will be reported.

Material and Methods

The giant keyhole limpet, *Megathura crenulata,* is obtained in fair numbers on the breakwaters and rocks at the entrance to Newport Bay and along the coast. They can be kept for a couple of months or more in running sea water aquaria. Sexually ripe individuals can be obtained at any season of the year, although they are relatively scarcer in the fall. The sexes cannot be distinguished externally. However, the animals can be "undressed" without injury and the gonads exposed to view. This is done by continuously stimulating, with a probe, the posterior edge of the mantle fold that covers the shell. The animal slowly retracts its mantle to beyond the edge of the shell which can then be raised slightly and the gonads examined through the transparent mantle that covers the viscera. The ovaries are dark green, the testes orange to creamy yellow in color.

The eggs and sperm are usually obtained by dissecting out the gonads, allowing them to shed in filtered sea water and straining through bolting cloth. Particularly in the case of the ovary the dissection involves injury to a small percentage (5 to 10 per cent) of the eggs. Occasionally the animals spawn in the aquaria. Sperm from the dissected testes contains clumps of "immature" cells which are not present in spawned sperm. The behavior of these clumps will be described below. Agglutinin solutions from naturally spawned and from artificially removed eggs show no difference in action on the sperm. It would be desirable, however, particularly for the purpose of purifying the agglutinin, to obtain spawned eggs, since, in the limpet, injury to some of the eggs is almost unavoidable when the ovaries are dissected out. However, attempts to stimulate spawning have thus far been unsuccessful.

The sex products can be obtained in relatively large quantities. Individuals weighing about 300 grams have, when ripe, gonads weighing about 15 to 20 grams, of which more than three-fourths are mature eggs or sperm. The sperm can be kept in a viable condition for as long as a week by removing the testes "dry" to a stoppered vessel and storing at 4° C.

The egg water is obtained simply by removing the supernatant sea water in which eggs have been standing. With concentrated suspensions it is generally necessary to pack the eggs down in a centrifuge. Extraction by solutions other than sea water will be described in a subsequent article. It may be mentioned here that the agglutinin is obtained in highest titer by extraction with pH 3 sea water or isotonic NaCl.

The Agglutination Reaction

The agglutination reaction in the keyhole limpet is readily visible macroscopically. When a drop of egg water is mixed with a small amount of a sperm suspension (Fig. 1*a*), the latter very quickly assumes the mottled appearance shown in Fig. 1*b*. This change can occur in less than five seconds when highly concentrated solutions of the agglutinin and strong sperm suspensions are employed. It is due to the formation of small spherical clumps of sperm. The clumps enlarge by fusion with one another (Fig. 1*c*) and, in a sufficiently strong egg water, a single agglutinate, containing practically all of the sperm and resting on the bottom of the dish, results (Fig. 1*d*). This latter condition can be reached within five minutes after the addition of the egg water. The single agglutinate will form even if the dish is left undisturbed after the initial mixing. It is generally circular in outline. In strong egg water the periphery usually has a perfectly smooth appearance rather than the somewhat irregular form shown in Fig. 1*d*. The size of the agglutinate depends, of course, on the amount of sperm employed. It also depends on the strength of the egg water. As the egg water is diluted the compactness of the clump first decreases. Then the number of initial agglutinates decreases and they fail to fuse into a single clump. Finally a dilution is reached at which no visible agglutination occurs. The time at which agglutination first becomes visible also varies with the strength of the egg water and the sperm suspension as will be shown below.

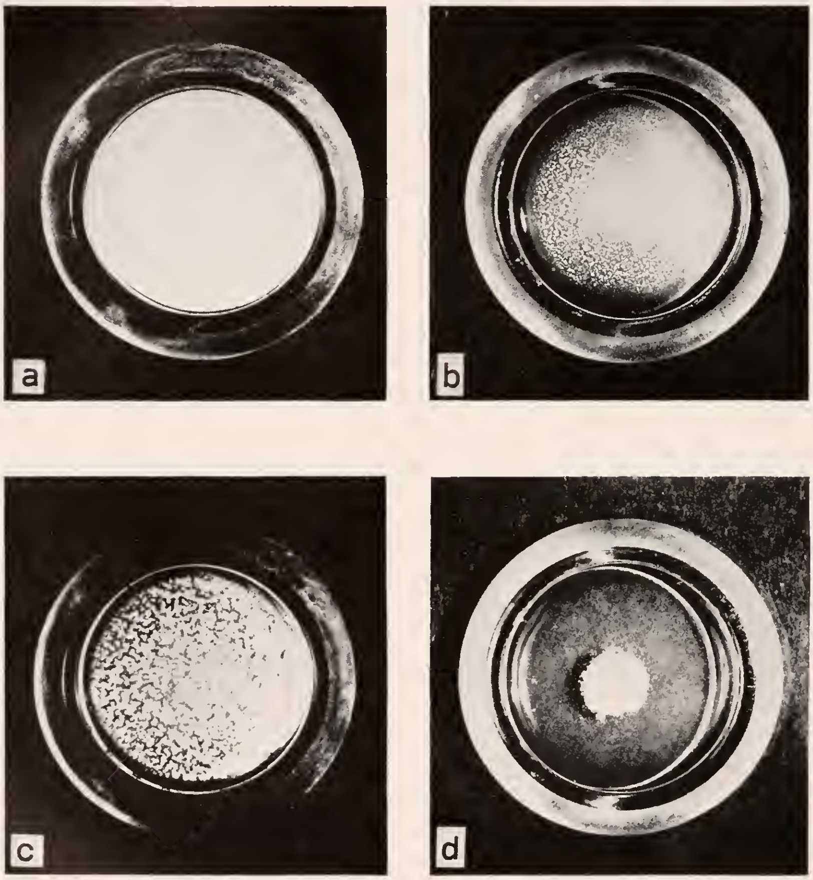

FIG. 1. Macroscopic appearance of the agglutination reaction in *Megathura*. Photographed in Syracuse dishes. × ⅔. *a,* Untreated sperm suspension (ca. 2 per cent); *b,* 15 seconds after addition of egg water; *c,* 30 seconds; *d,* 10 minutes.

The small agglutinates are typically spherical in shape. The larger ones are considerably flattened. The shape is similar to, although somewhat flatter than, that assumed by a drop of mercury in a dish of water.

The form of the agglutinates as seen under the microscope varies with the strength of the egg water employed and with the original condition of the sperm. In the following account an egg water will be designated as strong if, when mixed with an equal volume of a one per cent sperm suspension, it gives an agglutination reaction that is macroscopically visible in 10 seconds or less; moderately strong if visible in 10 to 60 seconds and weak if visible in more than 60 seconds.

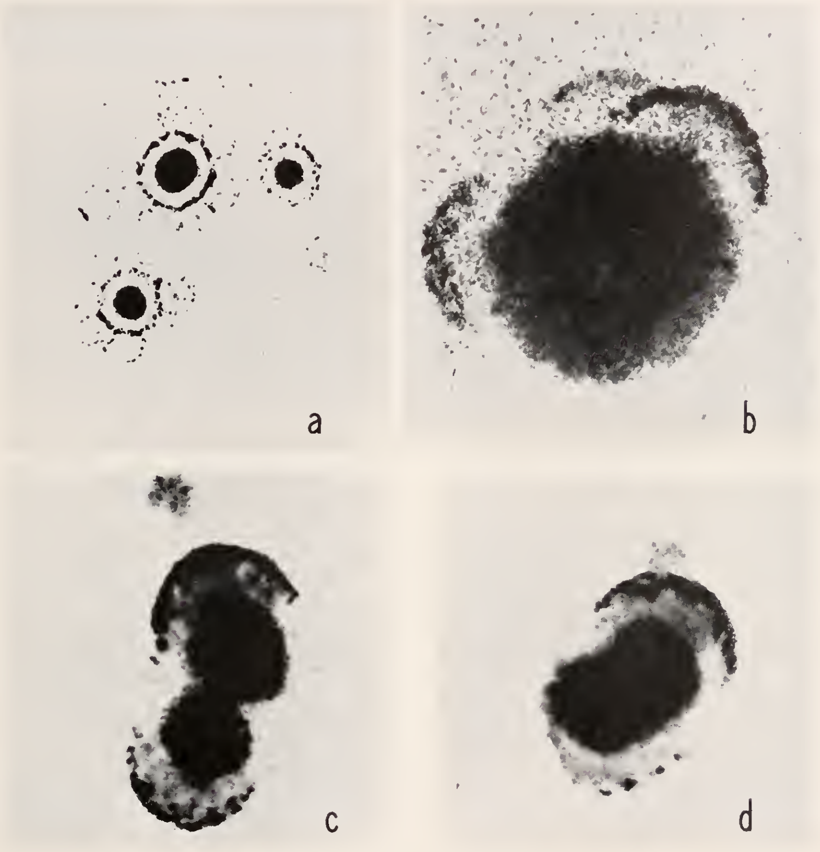

FIG. 2. Photomicrographs of sperm agglutinates of *Megathura*. *a*, Agglutinates formed in moderately strong egg water, showing spherical shell of sperm heads surrounding central mass of sperm. × 45. *b*, Agglutinate formed in strong egg water. showing shell segments derived mainly from sperm clumps of the type shown in Fig. 4*e*. × 150. *c* and *d*, Fusion of two agglutinates; *d* is taken 15 seconds after *c*. × 150.

Figure 2*a* shows, under low magnification, the type of agglutinates obtained with moderately strong egg water. They are composed of a spherical shell of sperm heads attached by their tails to a central spherical mass of sperm. In the smallest agglutinates the central mass is lacking, only the ends of the tails occupying the center. This is shown in Figs. 3*a* and *b*. The sperm heads of the shell are united in small clusters. When strong egg water is employed, a distinct shell does not form. In Fig. 2*b* an agglutinate in strong egg water is shown under high power. This figure shows three segments of a shell attached by the tails to the main mass of sperm. These segments arise from clumps of sperm initially present in the untreated sperm suspension (Figs. 4*d* and *e*). When such clumps are absent in the original sperm suspension, no shell segments are seen on the agglutinates produced in strong egg water. The sperm at the periphery of such " shell-less " agglutinates are attached by their tails to the central mass. They are extremely active, the heads moving rapidly in all directions as far as the tails allow. Only occasionally does a spermatozoön break away from the clump. In the smallest agglutinates formed in strong egg water, the center is occupied by the fused tails and the heads are independently and very actively motile. These agglutinates resemble very much the tridimensional pinwheels described by Sampson (1922) for the isoagglutination of sperm of the black chiton, *Katharina tunicata*. The " shell-agglutinates " can be converted into the " shell-less " type simply by the addition of some highly concentrated egg water. This also greatly increases the activity of the spermatozoa.

The agglutinates enlarge by fusion with one another. Figures 2*c* and *d* show the fusion of two agglutinates. When a partial shell is present they generally unite in the region where the shell is lacking, as shown in the figures. The presence of nearly complete shells retards, but does not prevent fusion and a single shell always results after the union. The shell itself does not increase appreciably in thickness, nor in distance of separation from the central mass even after the fusion of a great many agglutinates that have nearly complete shells. This must mean that after repeated fusions some of the sperm from the shells are withdrawn into the central mass.

Head and Tail Agglutination

Henle, Henle and Chambers (1938) have described agglutination reactions of bull sperm to antisera prepared in the rabbit that resemble very much the reactions described here. According to these investigators the bull sperm agglutinate head to head and tail to tail. By separating heads and tails, by absorption of antisera with heads or tails, and by immunizing separately with the parts they obtain strong evidence for the existence of specific head and tail antigens and antibodies. In the keyhole limpet, too, head-to-head and tail-to-tail union of the sperm can readily be found. However, the origin and behavior of such agglutinates indicate that only one kind of agglutinin is present in the original egg water.

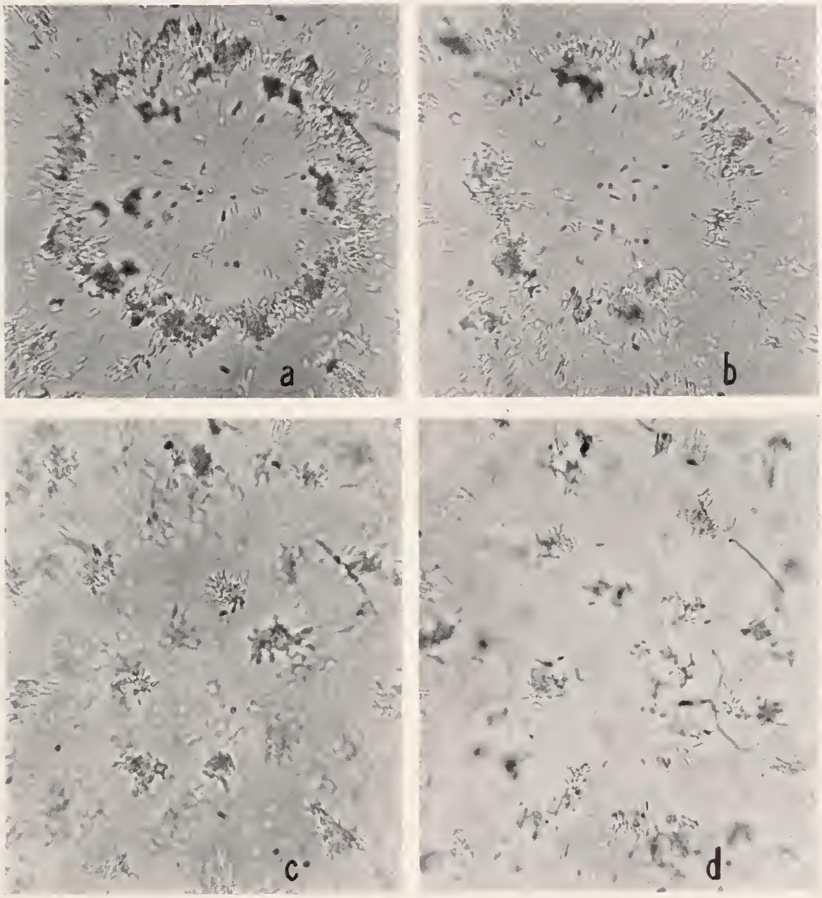

FIG. 3. Photomicrographs of sperm of *Megathura;* all × 300. *a* and *b*, Agglutinates formed in moderately strong egg water; taken before formation of central mass; shows tails agglutinated at center and heads clumped in groups (aggregated) at periphery. *c*, Agglutination in weak egg water; from each clump (aggregate) of sperm heads, bundles of tails (not distinctly visible) extend out in several directions and agglutinate with tails from other clumps. *d*, Aggregation reaction after dilution of sperm with sea water; the tails (indistinct) are not united with one another.

Two types of agglutinates showing both head-to-head and tail-to-tail union are found with keyhole limpet sperm. One type has been briefly described above (p. 164). It is obtained when a moderately strong egg water is employed. In the shells of the agglutinates, there are seen small clusters of about 20 to 50 heads. These are particularly distinct in the small agglutinates shown in Figs. 3*a* and *b* that lack the central mass of sperm. The head clumping disperses readily upon mechanical agitation and soon reappears upon standing. Addition of concentrated egg water causes the head clumping to disappear, as was mentioned above. In weak egg water no typical shells are formed. Bundles of tails extend out in several directions from the small clumps of heads, the tail bundles of different clumps uniting by their ends. Figure 3*c* represents that type of agglutination. The tails, however, do not show up well in the photographs due presumably to the smaller number present in the bundles and their irregular distribution in three dimensions. When only sea water is added to a sperm suspension a similar clumping of heads but without union of tails occurs as shown in Fig. 3*d*. This kind of behavior of sperm was originally described by Lillie (1913) in *Nereis* and is termed by him aggregation. The aggregation phenomenon has been shown by Lillie (1914) to be due to the CO_2 production of the sperm. The aggregates readily disperse upon mechanical agitation. Lillie showed that it was necessary for the sperm to be active in order to aggregate. Thus dilution with sea water increases the activity of the sperm and results in aggregation. Within the aggregates, however, the sperm are inactive. It is evident that the aggregation phenomena will account for the head-to-head clumping observed in the weak or moderately strong egg water. The egg water causes greater activation of the sperm than does dilution with sea water. It also gives more extensive aggregation. In strong egg water the keyhole limpet sperm remain intensely active for many hours. It is perhaps understandable then that aggregation (head-to-head clumping) should fail to occur. However, even after the sperm have quieted down (i.e. at the periphery of the agglutinates) in strong egg water there is no aggregation. The discrepancy may perhaps be explained by an interpretation that is offered for the aggregation phenomenon in the discussion part of this paper.

The other type of head-to-head and tail-to-tail "agglutination" that is found owes its origin to the presence, in the untreated sperm suspension, of clumps of sperm in which the heads are united. Figures 4*d* and *e* show two such clumps. They are composed of groups of about 50 to 100 spermatozoa attached firmly by their heads and with their tails radiating out separately. Upon the addition of egg water the ends of the tails agglutinate and the clump assumes one of the forms shown in Figs. 4*a*, *b* and *c*. The parachute-shaped agglutinate shown in Fig. 4*a*

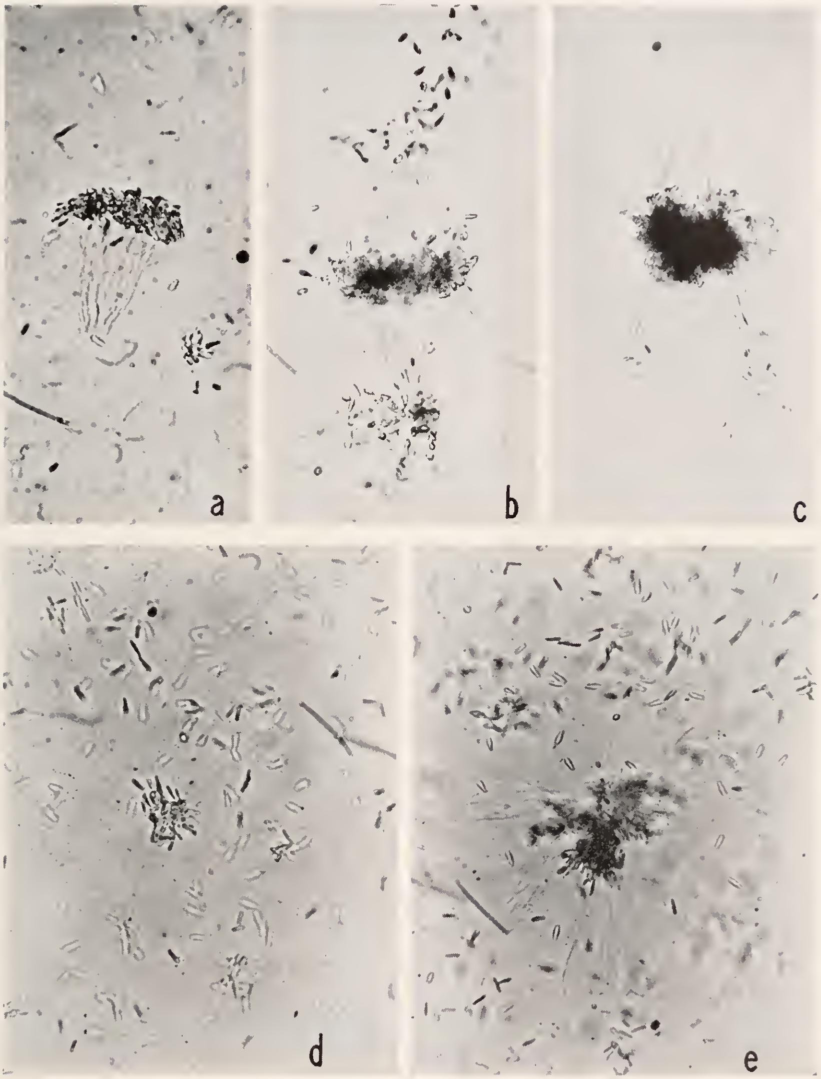

FIG. 4. Photomicrographs of sperm of *Megathura;* × 300; shows types of tail agglutination formed in: *a,* strong; *b,* moderately strong; *c,* weak egg water; from: *d* and *e,* "original" head clumps present in sperm suspension.

is obtained when strong egg water is added. The free tails come together on one side of the original clump of heads. With moderately strong egg water a great many of the agglutinates, that form from the original sperm clumps, have the spindle-shaped appearance shown in Fig. 4*b*. With weak egg water most of the agglutinates are of the type, shown in Fig. 4*c*, in which the tails come together at several foci. In other words, with increase in dilution of the egg water there is an increase in the number of foci at which the ends of the tails unite. The behavior of these "original" clumps parallels then the reaction of separate sperm to various dilutions of egg water.

These "original" clumps are not found in naturally shed sperm. Quite likely they consist of somewhat immature sperm that exude from the tubules that happen to be cut upon dissecting out the testes. The clumps cannot be broken up very readily by shaking the sperm suspension. Even in strong egg water the heads fail to separate and, as was mentioned above (p. 164), these clumps form the partial shells such as are shown in Fig. 2*b*. When they are very numerous they may form nearly complete shells. The number of these original clumps obtained in a sperm suspension varies with the condition of the testis and the amount of damage done in dissection. In a sperm suspension obtained from a very ripe male in which the testis was removed with care and allowed to shed undisturbed, haematocytometer counts showed one clump to 12,000 free sperm. A suspension obtained by shaking fragments of a small (not fully ripe) testis in sea water showed approximately 250 clumps to 12,000 free sperm. As the sperm suspension is kept the number of clumps gradually diminishes. Thus the last-mentioned suspension showed after five days storage at 4° C. 45 clumps to 12,000 free sperm.

The above evidence shows, then, that there is only one kind of agglutinin, namely tail-agglutinin, present in egg water of the keyhole limpet. The head-to-head unions, that are observed, arise in one of the two ways described above, namely from the "original" sperm clumps or as a result of the aggregation phenomenon. In neither of these is it necessary to assume the presence of a separate head agglutinin in the egg water. This conclusion differs, then, from that reached by Henle, Henle and Chambers (1938). It does not, however, necessarily conflict, since their experiments were performed with immune sera (produced by injection of bull sperm into rabbit) whereas the present experiments concern natural agglutinins.

Rate and Duration

The time at which agglutination first becomes macroscopically visible can be used as a convenient measure of the rate of the reaction. The rate measured in this way varies with the concentration of egg water and of sperm suspension. While in general the rate decreases with decrease in egg water concentration, the relation is not one of direct proportionality. Starting with a very strong egg water there is, on dilution, at first very little change in rate then a decrease roughly proportional to the dilution. With constant egg water concentrations, decrease in sperm concentration generally causes the rate to pass through a maximum. In addition, concentrated sperm suspensions may fail to give a visible reaction with certain egg water dilutions that do act on more dilute suspensions.

TABLE I

Time in seconds for agglutination to become macroscopically visible on mixing equal volumes of different concentrations of egg water and sperm suspension. Initial egg water = supernatant from a 10 per cent egg suspension, centrifuged after 2 hours. Initial sperm suspension = 30 per cent dry sperm = approx. 7.5×10^9 spermatozoa per cc. Temperature = 20° C.

rev = spontaneous reversal of agglutination within 24 hrs.
d = spermatozoa dead within 24 hours.

Egg Water Dilutions	Dilutions of Sperm Suspension							
	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
1	7	6	4	3	3	4	6 (*d*)	8 (*d*)
1/2	9	7	5	4	4	5	8	14 (*d*)
1/4	15	8	7	5	5	6	10	17 (*d*)
1/8	35 *rev*	14	10	7	7	10	15	20
1/16	105 *rev*	49 *rev*	21	14	13	17	28	30
1/32	—	—	140 *rev*	44	35	32	51	62
1/64	—	—	—	155 *rev*	190	150	140	110
1/128	—	—	—	—	—	420	300	260

These relations are shown in Table I, which contains the data of one of four similar experiments in which varying dilutions of egg water and sperm suspension were employed. As the data show, increasing the egg water concentrations for a given sperm suspension speeds up the reaction, but the increase in rate is greater with the dilute than with the concentrated egg waters. Decreasing the concentration of sperm for a given egg water at first speeds up the reaction, then retards it. Certain dilutions of egg water fail to give a reaction with concentrated sperm but do so with weaker suspensions. Thus 1/32 egg water does not give a visible reaction with undiluted or 1/2 diluted sperm; 1/64 egg water fails with 1/4 sperm, etc. This absence of a visible effect with certain ratios of the reactants is a familiar occurrence in serological reactions. It is known as the zone phenomenon (see Marrack, 1938; Zinnser, 1939). The non-

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agglutinating zone shown in the lower left corner of Table I corresponds to that of antigen (sperm) excess. The zone of antibody (agglutinin) excess cannot be determined macroscopically since it involves too great a dilution of the sperm suspension. It has, however, been noted microscopically that very dilute sperm fail to agglutinate in strong egg water.

In serological reactions the ratio of antigen dilution to antibody dilution at the points of most rapid flocculation is essentially constant. Here the ratio shifts from 1 : 8 (or 1 : 16) in the strongest egg water to 1 : 1 in the weakest. A number of factors may contribute to this divergence from a constant ratio. Thus the activity of the spermatozoa increases with dilution and also with increased concentration of egg water; the pH varies slightly with the concentration of sperm due to CO_2 production; and the ease of determining the beginning of agglutination varies with the sperm concentration due to the differences in opacity of the different suspensions. In view of these various factors there is no point at present in attempting a further analysis in terms of the zone phenomenon. The data show, however, that it is important to take into account sperm as well as egg water concentration in assaying the agglutinin. It may further be noted that sperm suspensions from different animals and of different ages will vary considerably in their reaction times.

The duration of the agglutination also varies with the relative concentrations of sperm and agglutinin. In general, with strong egg water and moderately concentrated sperm suspensions, the agglutination persists until after the spermatozoa die and disintegrate. With weak egg water and concentrated sperm suspension the agglutination reverses spontaneously. In the data that are given in Table I the cases in which spontaneous reversal occurred within 24 hours are marked *rev.* In all the other cases the sperm were still agglutinated at that time. In certain cases marked (*d*) in Table I the spermatozoa were all dead in 24 hours. This was determined by noting whether or not the spermatozoa became motile upon dilution with sea water. As may be seen in the table, it is in the more dilute suspensions that the spermatozoa are found to be dead in 24 hours. It is, of course, well known that spermatozoa die sooner in dilute than in concentrated suspensions. The data show that, in addition, egg water shortens their life. This is undoubtedly due to the fact that egg water increases the activity of the spermatozoa.

While in most of the combinations listed in Table I, agglutination had not reversed in 24 hours, there was a tendency in that direction in that the clumps became less compact and irregular. This was more pronounced the higher the ratio of sperm concentration to egg water. If the spermatozoa in the clumps are dispersed mechanically at any time while they are alive they will re-agglutinate. This occurs more rapidly the higher the ratio of egg water to sperm concentration. In those cases in which the agglutination reverses spontaneously, the time at which reversal occurs increases with increase in concentration of the egg water. It is difficult to obtain accurate data on the time of reversal as it is likewise difficult to obtain in those cases accurate times for the initial appearance of agglutination. This is due to the fact that an excess of sperm must be employed and, with dilution of the egg water, a smaller proportion is agglutinated. In the two cases of reversal listed in the first column of Table I, the approximate times are 5 hours for the $1/16$ egg water and 10 hours for the $1/8$ egg water. After spontaneous reversal, the addition of more egg water fails to cause re-agglutination or gives a weaker reaction than the control (untreated) sperm. In the latter instance the reaction is undoubtedly due to the presence of sperm that had not agglutinated initially.

In the sea urchin, Lillie (1913) showed that after spontaneous reversal the spermatozoa cannot be re-agglutinated. The present findings show that the reaction in the keyhole limpet is quite similar in that respect. But whereas in the sea urchin the agglutination lasts only a short time (up to about 15 minutes) and always reverses while the sperm are alive, in the keyhole limpet the reaction persists in most cases as long as the sperm are alive (24 to 48 hours) and only reverses in those mixtures where there is an excess of sperm.

Source of the Agglutinin

In the sea urchin, Lillie (1914, 1921) showed that the jelly layer surrounding the eggs contains the agglutinin in high titer. Loeb (1914, 1915) considered the agglutinin to be identical with the jelly and reported that it could not be obtained from eggs (*S. purpuratus*) in which the jelly had been removed by acid. Lillie, on the other hand, considered the agglutinin to be continuously produced by the egg. He reported its production by jellyless eggs of *Arbacia* and *Strongylocentrotus franciscanus,* and, although he confirmed Loeb's findings with *S. purpuratus,* he interpreted it to mean that the amount produced was too small to detect.

Repetition of the experiment of Lillie and Loeb on *S. purpuratus* again showed that after removal of the jelly no detectable amount of agglutinin is produced. It was also found that the acid sea water, which is employed to remove the jelly, extracts the agglutinin in very high titer and that this is not increased by allowing the eggs to stand in sea water for some time prior to extraction. One experiment illustrating this point may be cited. A suspension of *S. purpuratus* eggs in sea water was divided into three parts to which acid (0.6 cc. of 1 N HCl per 100 cc. of sea water → pH 3.5) was added after they had stood 5 minutes, 2 hours and 10 hours respectively. Samples of the supernatant sea water before the addition of the acid and of the neutralized acid sea water were tested on freshly diluted sperm. They gave the following times for the duration of agglutination:

Age of egg suspension	5 minutes	2 hours	10 hours
Reaction before acidification	no aggl.	3 to 4 minutes	7 to 9 minutes
Reaction after acidification	15 to 16 minutes	15 to 17 minutes	14 to 16 minutes

While in ordinary sea water the agglutinin titer increases, the acid sea water removes no more agglutinin after 10 hours than at the start. After removal of the acid sea water no agglutinin could be obtained from the eggs even after 24 hours standing in sea water. In another sample of the same eggs, the sea water was removed after 10 hours and acid sea water added. The acid sea water gave in this case a reaction lasting 8 to 9 minutes, while the control gave, as before, 7 to 9-minute reactions. There is then no evidence for the secretion of agglutinin by the eggs of *S. purpuratus*. It must either all be present in the jelly at the start or must be the jelly itself. As is well known, the jelly slowly dissolves as the eggs stand in ordinary sea water and this would account for the increase in agglutinin titer of the supernatant.

The jelly of the sea-urchin egg can also be dissolved by means of another agent, namely the proteinase chymotrypsin. This enzyme in a concentration of 1 per cent dissolves the jelly within about 15 minutes. At the same time it completely inactivates the agglutinin. After the treatment no agglutinin production could be detected. Again it appears that the agglutinin is the jelly or something intimately associated with it.

In the keyhole limpet the evidence points in the same direction. The jelly of these eggs dissolves much more slowly in acid sea water than does that of sea-urchin eggs, but by centrifuging in the acid solution jellyless eggs can readily be obtained. These when allowed to stand in sea water produce no detectable agglutinin. In the acid sea water the agglutinin is obtained in very high titer. The same type of experiment as described above for the sea urchin was performed on the keyhole limpet eggs, with the following results, for the time at which visible agglutination appears on testing the solutions.

Age of egg suspension	5 minutes	1 hour	2 hours
Reaction before acidification	4 to 6 minutes	30 seconds	12 seconds
Reaction after acidification	7 to 8 seconds	7 to 8 seconds	7 to 8 seconds

As before, the agglutinin titer (the time for visible agglutination decreases with increase in agglutinin concentration as shown in Table I) of the supernatant sea water increases with the time that the eggs remain in contact with it. There is, however, no corresponding increase in the amount of agglutinin that can be obtained after adding the acid. This remains essentially constant showing again no production of agglutinin by the eggs.

While the above evidence locates the agglutinin in the jelly layer surrounding the egg, it does not identify it with the jelly. To determine this point it would be necessary to know how many substances comprise the jelly. It seems unlikely that more than one is involved, but in the absence of direct information it may only be concluded at present that the agglutinin is a component of the jelly layer.

Specificity

Lillie (1919) and Just (1930) have shown that sperm agglutination is both tissue- and species-specific. In certain instances (e.g. *Arbacia* egg water or blood on *Nereis* or *Echinarachnius* sperm) cross reactions were obtained. It was noted in such cases that the reaction fails to reverse spontaneously and the term hetero-agglutination was used to designate the cross reactions. Since in the keyhole limpet the iso-agglutination also fails to reverse spontaneously under most conditions and resembles in this respect hetero-agglutination, it was of interest to examine the specificity of the reaction.

Tests were made with the blood and with sea water extracts of the foot, mantle and viscera of both male and female keyhole limpets. The extracts were prepared by washing the fresh tissues, by grinding them and by first freezing at — 70° C. in sea water. In no case was agglutination of sperm obtained.

The egg waters of several different animals were tested on keyhole limpet sperm, and at the same time egg water of the keyhole limpet was tested on the foreign sperm. The animals examined were *Haliotis cracherodii* (abalone), *Astraea undosa* (top shell), *Lottia gigantea* (limpet), *Ischnochiton magdalenensis* (chiton), *Urechis caupo* (gephyrean worm), *Strongylocentrotus purpuratus* (sea urchin), *Dendraster excentricus* (sand dollar) and *Patiria miniata* (starfish). None of these showed either agglutination of keyhole limpet sperm with their egg water or agglutination of their sperm with keyhole limpet egg water. Of these animals the first two belong to the same division (rhipidoglossa) as the keyhole limpet. No animals belonging to the same family were available for testing. It would, of course, be of considerable interest to

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examine more closely related animals in order to determine the extent to which the reaction is specific. The present results make it clear, however, that although there may be a superficial resemblance to what has been described as hetero-agglutination, the reaction in the keyhole limpet is tissue specific and at least to some extent species specific.

Discussion

As the evidence presented in the first part of this paper shows, there is present in egg water of the keyhole limpet only one kind of agglutinin, namely that for the tails of the sperm. The clumping of heads that occurs in all but the strongest egg water is evidently an aggregation phenomena. Aggregation, as Lillie (1913) first noted, occurs upon dilution of a sperm suspension. The addition of egg water leads to aggregation presumably for the same reason that dilution does, namely as a result of the increased activity of the spermatozoa.

Lillie showed that the aggregation reaction can be produced by the addition of CO_2 to the sperm suspension, but not if the CO_2 tension is too high, and that it fails to occur in sperm suspensions to which alkali has been added. He (1919, p. 103) interpreted the reaction as a chemotactic response to CO_2. It is, however, difficult to see how the necessary CO_2 gradient would be produced and maintained in a suspension of actively moving spermatozoa. Furthermore the sperm appear to be actually stuck to one another in the aggregates.

In view of these considerations, the following alternative hypothesis may be suggested for the mechanism of the aggregation reaction. The increased CO_2 production resulting from the increased activity of the sperm lowers the pH of the sea water and thereby produces some change on the surface of the sperm head that enables them to stick to one another when they meet. This change might be regarded as a general increase in stickiness. The sperm do not, however, show an increased tendency to stick to any object but rather only to one another. It seems preferable, then, to consider that the slight increase in acidity causes a partial dissociation of some surface material (S) of the sperm from the underlying substance (U) with which it is in loose combination. This would produce areas on the sperm head where U is exposed and capable of uniting with S on other sperm. In this manner clumps of several sperm heads could be formed. To explain the failure of aggregation to occur in strong egg water (p. 166), it would be necessary to assume that the greater activity of the spermatozoa caused a sufficient lowering of the pH to give complete dissociation.

The view presented here represents an extension of the lattice theory of agglutination (Heidelberger, 1938; Marrack, 1938). The agglutinin here is assumed to be the surface material, *S*, initially present on the head of the sperm. The aggregation phenomenon is, then, regarded as an auto-agglutination reaction. It is attributed to a partial dissociation of the agglutinin occasioned by change in pH (or perhaps other factors as well) and combination of the underlying material with agglutinin present on other spermatozoa. The recent results of Southwick (1939*b*) with *Chiton* sperm could be interpreted on this basis. Auto-agglutination has been often described in bacteria and blood cells, but as far as the author is aware, no interpretation of this kind has been offered for the phenomena. To test the hypothesis it would be necessary to obtain from a particular kind of cell a substance capable of agglutinating cells of the same kind. We have not succeeded as yet in doing this with the sperm. With eggs of the sea urchin, however, we have been able to obtain evidence of this sort. That is, a substance can be extracted from the eggs that is capable of agglutinating eggs. This work will be reported in a subsequent article. It is mentioned here, however, as support for the interpretation suggested for the aggregation (auto-agglutination) reaction in sperm.

Heidelberger and Kabat (1936) report an experiment which, I believe, further supports this view. They " sensitized " bacteria (Pneumococcus I M) by adding excess agglutinin, and resuspended the coated cells in fresh saline. The addition of untreated cells of the same type causes the entire mass of cells to agglutinate. This does not occur when cells of other types are added.

In regard to the spontaneous reversal of agglutination, the keyhole limpet reaction evidently differs only in a quantitative manner from that in the sea-urchin. Thus under most conditions the agglutination in the keyhole limpet persists until the sperm are dead. Some further observations may be mentioned here that correlate with this difference in the two forms. A substance (anti-agglutinin) has been extracted from sea-urchin sperm (Frank, 1939; Southwick, 1939*a*) and from keyhole limpet sperm (Tyler, 1939) which has the property of neutralizing the agglutinin of eggs of the same species. When solutions of agglutinin and anti-agglutinin are mixed a precipitate is formed. In the sea urchin this occurs within about 2 to 10 minutes depending upon the concentrations. In the keyhole limpet the precipitate does not form until after 20 or more hours. Neutralization of the agglutinin, however, occurs almost immediately after addition of the anti-agglutinin in both species. The time of precipitate formation corresponds roughly to the time at which agglutination reverses in the two forms. It appears, then, that reversal is due to some secondary change in the compound formed by the combination of agglutinin with anti-agglutinin on the sperm, and that this secondary change is analogous to the precipitate formation observed in the test tube on mixing solutions of agglutinin and anti-agglutinin.

In the sea urchin Lillie (*op. cit.*) showed that after spontaneous reversal the spermatozoa cannot be re-agglutinated. The same is true for the keyhole limpet as was noted above. Lillie (1913, p. 558) also noted a decrease in the fertilizing power of sperm suspensions after agglutination and reversal. I have confirmed and extended this with the sea urchin as well as with the keyhole limpet. These results, the details of which will appear in a later publication, show that sperm are no longer capable of fertilization although they are still highly motile after reversal of agglutination. It seems likely that the loss of the capacity for agglutination and for fertilization is due to the secondary change mentioned above. This would involve a change in the nature of the "reacting" surface of the spermatozoa either by removal of the reactive material, as Lillie proposed, or by the presence of altered agglutinin on the surface.

In the account of the agglutination reaction given in the first section of this paper (p. 162) it was pointed out that the form of the agglutinates is that of drops of a heavy liquid immiscible with water. Furthermore, separate agglutinates fuse with one another in very much the same manner as do liquid drops. These considerations lead, then, to the view that the initial reaction of agglutinin with the reactive material (anti-agglutinin) on the sperm causes the agglutinin to become insoluble in sea water and to separate out as a liquid. If such a change occurred in the agglutination reaction, one might expect to see it in the precipitin reaction; that is, on mixing solutions of agglutinin and anti-agglutinin. However, although there is an immediate neutralization of the agglutinin, no visible change occurs until some time later when the precipitate forms. No separation into two layers, as might be expected with two immiscible liquids, has as yet been seen. This does not necessarily rule out the postulated change since the conditions of the precipitin reaction might be such as to form very fine emulsions, whereas in the agglutination reaction the liquid might separate out from the sea water more readily due to the activity of the sperm, or the fact that the anti-agglutinin (on the sperm) is for the most part not in solution, or to other possible factors. Thus, while it seems almost essential, in order to interpret the form and behavior of the agglutinates, to assume that the agglutinin becomes an insoluble liquid, there is at present no direct evidence for or against that view.

Summary

1. Egg water of the keyhole limpet, *Megathura crenulata,* causes a strong agglutination of the sperm of this species. In contrast to the rapid spontaneous reversal that occurs in the sea urchin, the reaction here is relatively irreversible, the agglutinates persisting in most cases until after the death of the sperm. Spontaneous reversal occurs when an excess of sperm is employed.

2. When anti-agglutinin (sperm extract) is added to agglutinin (egg water) the latter is immediately neutralized and a precipitate later appears. The precipitate appears very much later with the keyhole limpet substances than with those of the sea urchin, the time corresponding roughly to that at which reversal of agglutination occurs in these two forms.

3. With varying concentrations of agglutinin and sperm suspension the occurrence and rate of agglutination behaves in a manner similar to that described as the zone phenomena in serological reactions.

4. The sperm agglutinate by their tails and under certain conditions by their heads as well. But the evidence shows only tail agglutinin to be present in the egg water, the head clumping being ascribed to the aggregation reaction which the sperm exhibit on dilution with sea water. The aggregation reaction is considered as an auto-agglutination and a new interpretation for this is offered based on the partial dissociation of a substance from surface of the sperm head as a result of change in pH or other conditions.

5. The agglutinin is shown to be a component of the jelly layer of the egg. No evidence of its continuous production by the egg was obtained in the sea urchin or in the keyhole limpet.

6. Although the reaction bears a superficial resemblance to what has been described as hetero-agglutination, it is found to exhibit both tissue and species specificity.

7. The agglutinates have the form and behavior of liquid drops. It is suggested that this is due to the agglutinin forming an insoluble liquid upon reaction with the spermatozoa.

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MOULTING, GROWTH, AND SURVIVAL AFTER EYESTALK REMOVAL IN UCA PUGILATOR

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Following the discovery of a chromatophorotropic hormone by Perkins (1928) and more especially, following the discovery of the sinus gland and other endocrine glands in the crustacean eyestalk by Hanström (1933–1937) and others, considerable interest has been shown in the extra-chromatophorotropic functions of the eyestalk. Hanström (1937) has pointed out that the endocrine glands discovered by him are present in various crustaceans which have either no chromatophores or show no metachrosis. Nevertheless, extracts prepared from the eyestalks of such animals are effective on the chromatophores of standard test animals. On these grounds, Hanström has suggested that the chromatophore hormone(s) may have other and more significant functions. It is also equally possible, however, that the eyestalk produces other hormones (besides the chromatophore activating system) which have significant functions in the life of the crustacean.

Among the various effects attributed to the eyestalks as based on experiments of either eyestalk extirpation or of the injection of eyestalk extracts, the relation between the eyestalks and the moulting process of crustaceans is of great interest. In our study of the specificity of the chromatophorotropic hormone of *Uca* (Abramowitz and Abramowitz, 1938), we observed that animals deprived of their eyestalks moulted more frequently than normal animals. We further remarked that the eyestalks did not seem to be essential to the life of this crustacean because we had maintained some blinded animals throughout the summer months (11 weeks in all) at the end of which time the experiments were discontinued. We have returned to these observations in order to obtain some quantitative data concerning these relationships. We were also stimulated to study in detail the effect of eyestalk removal on the viability of *Uca* because of the results obtained somewhat later by Brown (1938) on the crayfish, *Cambarus,* whose eyestalks are essential to its continued life.

The chronological development of this subject is quite interesting because the effects of eyestalk removal on moulting were known long before they were fully appreciated. Perhaps the first observation in this connection was that of Megašur (1912) who noted that the first and subsequent moults occurred earlier in blinded *Astacus vulgaris* than in normal animals. Koller (1930) found that less calcium was deposited in the exoskeletons of blinded animals than in those of normal animals. We (1938, 1939) also noted the moult-inhibiting function of the eyestalks of *Uca pugilator,* which moults more frequently following loss of its eyestalks. Hanström (1939) observed that the first moult took place earlier in blinded *Eriorcheir sinensis* than in normal specimens. Brown and Cunningham (1939) have established this relationship on a glandular basis for they were able to lower the high percentage of moulting in eyestalkless *Cambarus* by implanting the sinus gland. The literature on the viability effect is less uniform. Various investigators working with decapod crustaceans whose eyestalks had been removed reported survivals ranging from a few months to years (Megašur, 1912; Herbst, 1901; Abramowitz and Abramowitz, 1938). On the other hand, *Cambarus* succumbs within a few days following eyestalk amputation (Brown, 1938).

Materials and Methods

The experimental material consisted of two series of animals, one comprising slightly over a hundred specimens, and the other of 753 specimens. In the first experimental series, one hundred individuals (*Uca pugilator*) consisting of a mixed population of both sexes and of various sizes were blinded by excision of their eyestalks and placed in large crystallizing dishes for 4 or 5 days. After the fourth day, 76 animals, chosen at random, were segregated into 76 paper cups. Each animal was segregated because *Uca* is cannibalistic even if well fed, and is especially cannibalistic if allowed access to moulting individuals. Twenty-five unoperated animals were also segregated and maintained as controls. Both sets of animals were fed daily with small pieces of clam. Sea water, which was placed in the cups to a depth of about one-quarter of an inch, was changed daily. The experiment was continued for 48 days during which time observations on moulting, growth, and viability were made.

The second series (753 blinded animals) was used to check the rate of moulting found in the first series. The animals were maintained in large crystallizing dishes, about 30 specimens per dish. These animals were fed and given fresh sea water daily. No observations on viability were made in this series because many of the animals were devoured during ecdysis. This series was carried for only 30 days.

RESULTS

Operative Mortality

The operative mortality was quite low in spite of the fact that the eyestalks were merely cut off with a pair of scissors without further measures. The usual operative mortality is 8 per cent, although it may rise to 12 per cent. All deaths occurring during the first 4 days following the operation were listed as operative deaths. In the large series of 753 animals, 8.7 per cent died within the first three days. After the first few days, the animals recover from the shock of the operation, and appear to be in good condition. They are vigorous, eat well, and show, at least superficially, no signs of serious weakness. Such a condition is maintained until the first moult, after which serious debilities appear. Some of these post-operative animals die at various times before their first moult, but these cases will be analyzed in another section. No observations were made with regard to a diurnal cycle of motor activity.

Moulting

In the first series of 76 animals, the first case of moulting occurred on the eleventh day following excision of the eystalks. By the sixteenth day, 50 per cent of the animals had moulted, and by the thirty-fifth day, all of the operated animals had moulted. The control group of animals showed quite different results. Only 7 of these animals had moulted during the 48 days of observation, the first case occurring on the twenty-fifth day after the experiment was started. The results are expressed in the form of curves (Fig. 1), showing the percentage of animals moulted at daily intervals following the operation. Such curves bring out more readily the accelerating effect of eyestalk removal on moulting.

The larger series of 753 animals showed a moulting rate essentially similar to that already described. The shape of the curve was approximately the same as that in Fig. 1, but was shifted farther along on the abscissa. The first moult occurred on the ninth day but the rate did not rise until the fifteenth day when 10 per cent of the animals had moulted. From this point on, the curve rose parallel to that in Fig. 1 but separated from it by a distance equal to 4 days on the abscissa. Thus, 25 per cent of the isolated crabs moulted on the thirteenth day whereas 25 per cent of the animals in the second series moulted on the seventeenth day. Fifty per cent of the isolated animals moulted on the sixteenth day whereas the same percentage of the non-segregated crabs moulted on the twentieth day. This difference became more noticeable, for only 80 per cent of the animals had moulted on the twenty-ninth day, as compared

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with the twenty-first day for the segregated animals. We are not in a position to state the cause of this difference in the time relations of moulting in segregated and non-segregated animals, but we wish to point out that a sufficiently large group of animals has been used to demonstrate the accelerating effect of eyestalk removal on ecdysis.

The percentages of moults (Fig. 1) were calculated on the basis of 70 animals, since 6 of the animals died before they had moulted. Only the first moults were included in this curve. Three of the animals moulted twice during the experiment, the second moult occurring between the twenty-first and twenty-sixth day after the first moult. Only 29 per cent of the control animals moulted during the 48 days, and none of these animals moulted a second time. Excluding the 3 cases in which moulting occurred twice, the degree of ecdysis in the operated group is about 300 per cent that of the control group over the same period of time. Furthermore, the onset of moulting in a mixed population chosen at random is two weeks earlier in the operated group than in the unoperated group. Finally, the loss of the eyestalks may shorten the inter-moult period. The inter-moult interval, of course, was not determined since none of the normal specimens moulted twice during the period of observation. However, a shorter inter-moult interval may result from eyestalk removal since 3 of the operated animals moulted twice (between 21–26 days following their first moult) during the 48 days. The unoperated moulted animals were carried for only 20–23 days after their first moult and consequently it is not possible to decide whether these animals might have moulted within a short time after the experiment

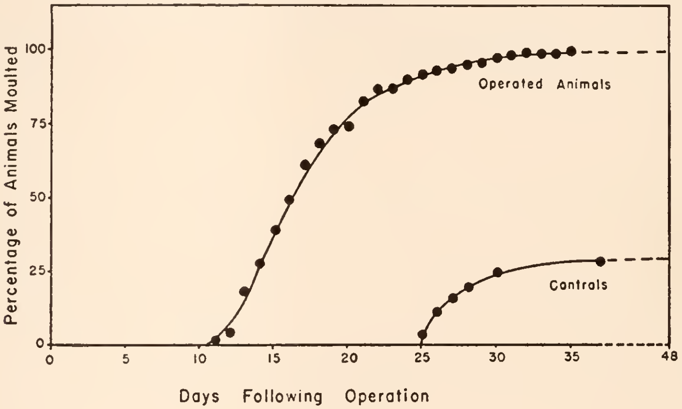


FIG. 1

was discontinued. We feel, however, that the normal inter-moult period must be longer than 3–4 weeks since in a mixed population most of the animals would have moulted within this time. Yet only 29 per cent of the animals had moulted in 7 weeks.

Viability

The relation between viability and moulting is quite complex, as an analysis of our data will indicate. Brown (1938) has presented evidence for the existence of a chemical substance that is essential to the life of the crayfish, and Brown and Cunningham (1939) further suggest that the viability function of the eyestalks is different from the moult-inhibiting function. The basis of the latter is the fact that blinded animals sometimes continue to live several days after moulting and then die.

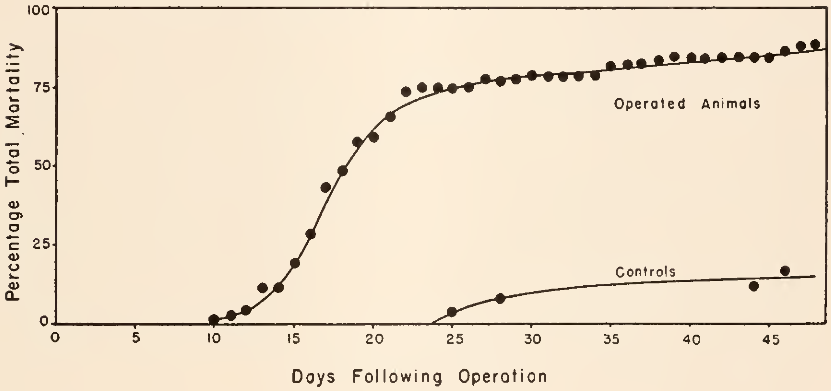


FIG. 2

The total mortality in the control and operated groups of the first series of animals is shown in Fig. 2. The mortality curve is strikingly similar to that of the moulting curve, and gives the superficial impression that death and moulting bear a causal relationship. However, the analysis of the individual case histories reveals a more complex situation, and brings out more significant aspects of this relationship than is shown by a total mortality curve.

In the first series of animals, 7.9 per cent of the operated animals died directly without having moulted. In the second, larger series of animals, only 3.2 per cent succumbed before moulting. These deaths occurred from the tenth to thirtieth post-operative day. We regard these deaths as due directly to the loss of the eyestalks, since no complicating processes had intervened between operation and death. This mortality percentage is much too low to establish the presence of a life-sustaining secretion by the eyestalks, especially since 8.5 per cent of the unoperated specimens died in the same fashion. We conclude, therefore, that the loss of the eyestalks *per se* is not a cause of death in the species. As pointed out previously, many of the operated animals are in good shape for as much as 30–35 days but succumb shortly after moulting. If we exclude the 7.9 per cent of the animals dying before moulting, we can then state that mortality is coincident with moulting.

The total mortality over 48 days was 89 per cent in the operated group and 16.6 per cent in the control group. There can be no question, therefore, that the eyestalks are concerned in some way with viability. Since 8 per cent of the operated animals died before ecdysis, the total mortality during or some time after moulting is 81 per cent. Such a classification may not be justifiable for we cannot assume what the mortality might be provided the operated animals did not moult. Nevertheless, this division reveals a significant fact—that 91 per cent of all deaths occur during or shortly after moulting, and that only 9 per cent of the total deaths occur before moulting even though this latter takes place over a month. Correlated with these figures is the observation that the animals show no signs of impending death until after they have moulted in spite of the fact that some animals do not moult until a month or so after the operation. In the control group, half of the total deaths occurred before moulting, and half afterwards.

Nevertheless, 10.5 per cent of the total operated animals survived throughout the entire experiment. This constitutes 11.4 per cent of those animals which had moulted either once or twice, or 10.4 per cent of those animals which had moulted only once. On this basis we can also state that the eyestalks of this species have a function in addition to moult control as Brown and Cunningham (1939) have found for *Cambarus*. Thus, no definite answer can be given to the question of the separate control of moulting and viability in *Uca,* and the best we can do for the present is to give an estimate by a further analysis of our individual case histories of the relative proportions of a possible direct or indirect control of viability by the eyestalks.

The following table shows the percentages of 67 animals that moulted once and the time in days after moulting at which they succumbed:

Percentage	Days after moult
70	during or within 1 day
4.5	3
3.0	4
3.0	5
1.5	8
1.5	14
1.5	20
1.5	23
1.5	27
1.5	31

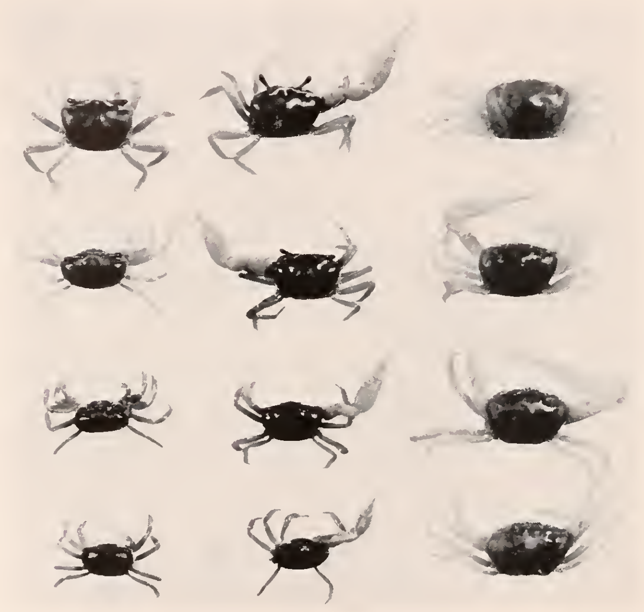
Of the animals which moulted twice, two died during ecdysis and one continued to live. Thus, about 75 per cent of the moulting deaths may be described as being due directly or indirectly to the process of ecdysis, while 25 per cent (which includes the viable 10.5 per cent) of the animals do not fall within this category.

Finally, another way of analyzing the data may be helpful. Part of the total moulting mortality, we feel, is due to mechanical difficulties in ecdysis, and part, probably a major portion, is due to some debility whose appearance is concomitant with or aggravated by the moulting process. We repeatedly observed that the animals have difficulty in moulting under the described environmental conditions. They appeared to have no difficulty in detaching themselves from the walking appendages and ventral abdominal segments, but the animals were frequently unable to remove themselves from the carapace and especially from the mouth parts. The scar tissue of the eyestubs also appeared to offer a mechanical difficulty. That two of the normal animals that moulted, or 28 per cent, also died during moult indicates the possible existence of such a mechanical factor which probably does not exist in the normal habitat. The remaining deaths are undoubtedly due to some severe deficiency. This must be true because 60 per cent of the animals which moulted once successfully, succumbed nevertheless in a weakened condition of a varying duration of time after moulting. Such a deficiency is not seen in the normal specimens, for those (72 per cent) which moulted successfully continued to live and were indistinguishable from normal specimens which had not moulted.

Growth

Since eyestalk removal accelerates moulting, it was expected that eyestalkless animals would attain a larger size. Due to the paucity of the animals which survived the 48-day experiment, we did not keep individual records of weights. However, the few animals which survived, especially one which had moulted twice, were virtually gigantic crabs.

In order to compare the experimental and normal specimens, we picked out the largest male and female specimens from a lot of 500 normal animals. These are shown in Plate I, together with the ordinary sized, and small specimens. The experimental specimens are also shown in Plate I. A glance at Plate I leaves little doubt that removal of the eyestalks leads to increased size.



Explanation of Plate I

The two columns at the left comprise 4 sets of normal animals chosen from a lot of 500 specimens, showing two intermediate stages from the largest male and female crabs to the smallest. The third column (at the right) shows 2 sets of animals whose eyestalks had been removed 48 days previously. All specimens are one-half natural size.

Miscellaneous Effects of Eyestalk Removal

Animals deprived of their eyestalks seem to lose pigmentation, an effect which becomes evident especially after moulting. Normal animals have a blackish carapace in which purple and red colors may also be seen. After eyestalk removal, the entire animal pales noticeably—a well-known effect due to the concentration of melanophore pigment. The carapace of blinded animals is much paler than that of a normal specimen, though not the cream yellow color of the walking legs. Although there is considerable variation, the shade assumed by the carapace may be described as blackish gray. After moulting, the carapace assumes a grayish-white appearance, which may be due to the loss of pigment. The moulted animal sometimes gives the appearance of an albino specimen. Either pigment has been lost, or sufficient replacement has not taken place after ecdysis. More cannot be said until further study.

Uca breeds during September, as indicated by the appearance of large masses of eggs, copulation, and finally shedding of the eggs. Animals without eyestalks have been observed to copulate and shed their eggs. However, such animals were blinded for only a few weeks before the onset of the breeding season and thus sufficient time may not have elapsed for any effect on reproduction to take place. This is worth investigation, however, for as yet no endocrine influence on the reproductive system of crustaceans has been demonstrated.

SUMMARY

After removal of the eyestalks in *Uca pugilator,* all of the animals moulted within 35 days after the operation, and a few of the animals moulted twice within 48 days. Only 29 per cent of the normal animals moulted within 48 days, and none moulted twice. Eyestalk removal not only accelerated moulting but shortened the time in which the first and second moults occurred. The time at which the same percentage of moulting occurred was somewhat greater for non-segregated than for segregated animals.

Viability is related to moulting, and most of the deaths following eyestalk removal in this species are due in part to a mechanical difficulty and in part to some severe deficiency occurring during or aggravated by ecdysis. There is insufficient evidence to decide whether the eyestalks secrete a specific, life-sustaining hormone directly responsible for the continued life of the animal, or whether the viability effect is in some way dependent on the moult-inhibiting function of the eyestalk. We do not believe that the loss of the eyestalks *per se* is directly responsible for the total mortality seen in eyestalkless crabs because of the negligible percentage of deaths occurring from 1 to 5 weeks before moulting, and because operated animals live for 5 weeks without showing any external signs of serious debilities only to succumb after ecdysis. Seventy-five per cent of the total mortality can be related directly or indirectly to ecdysis, while 25 per cent (which includes the 10.5 per cent viable specimens) does not fall in this category and may be cited in support of a differentiation of the viability and moult-inhibiting functions of the eyestalk.

Eyestalk removal also leads to increased size. Some of the eyestalkless specimens 48 days after the operation have attained gigantic proportions. Pigmentation also seems to be lost, an effect which is noticeable after the first moult. No effect of eyestalk removal has been noted on copulation and shedding of eggs.

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THE ORIGIN OF THE DIABETOGENIC HORMONE IN THE DOGFISH[1]

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It is generally regarded that the anterior lobe of the pituitary produces a diabetogenic hormone. A good deal of the evidence for this view has been obtained by injection of extracts of the various lobes of the pituitary into normal or hypophysectomized-depancreatized animals. Although posterior and intermediate lobe extracts have been found to be mildly diabetogenic, most of the diabetogenic activity is found in the extracts of the anterior lobe (Houssay, 1937). Recently, an attractive possibility has been presented by O'Donovan and Collip (1938) to the effect that the diabetogenic, ketogenic, and specific metabolic effects of pituitary extracts represent the action of the melanophore hormone, intermedin. This view not only tends to reduce the number of hypophyseal principles but also supplies a function for the melanophore hormone in warm-blooded animals. The evidence for this view is mainly of a chemical nature, since extracts prepared from the pituitary in various ways, or from the intermediate lobe alone, produce the various metabolic effects mentioned above and at the same time are rich in the melanophore hormone.

If intermedin is responsible for the diabetogenic effects, it should be possible to obtain supporting evidence by physiological methods in some cold-blooded vertebrate that depends on the pituitary for the regulation of its integumentary melanophores. A very favorable animal for such an investigation is the smooth dogfish, *Mustelus canis*, in which Orias (1932) has already demonstrated the amelioration of pancreatic diabetes by complete hypophysectomy. The physiology of the intermediate lobe of the pituitary of this animal is well understood, and its secretion can be controlled at will (cf. Abramowitz, 1939*a*). The effects of the presence or absence of intermedin in the blood on the resting blood sugar, or on the severity of experimental diabetes, could then easily be determined. Of considerable interest in this connection is the report of Slome (1936) that the blood sugar of black-adapted toads is significantly higher than

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[1] Aided in part by a grant from the Rockefeller Foundation.

[2] Contribution No. 246.

that of white-adapted toads, a situation which is not unfavorable to the view that intermedin is diabetogenic. However, Slome concluded that the diabetogenic hormone was produced by the anterior lobe since removal of the entire pituitary or of the anterior lobe alone decreased the severity of pancreatic diabetes. Another advantageous feature in using the dogfish is the facility of surgical treatment of the pituitary. Either the anterior or the neuro-intermediate lobe can be removed separately, and it is therefore possible to determine the relative effects of different parts of the hypophysis on carbohydrate metabolism.

The experiments to be described in this paper were undertaken to determine whether intermedin is identical with the diabetogenic hormone. The richness of intermedin in partly purified extracts possessing the ability to produce metabolic effects is suggestive but rather circumstantial evidence that such effects are due to intermedin, since many chemically similar hormones may be present in partly refined preparations. This question can be tested directly in the dogfish by experimental regulation of intermedin secretion. If the chromatic function of the pituitary can be correlated with its diabetogenic function, the probability that both effects are due to the same hormone is enhanced. On the other hand, if these two effects can be shown to occur independently, it becomes reasonably certain that they are not due to the same principle.

Materials and Methods

Pups, yearlings, and two-year-old dogfish, *Mustelus canis,* of both sexes were employed since sufficient numbers of one age group were not obtainable. However, most of the animals were yearlings. The fish were captured either by large traps or caught on hook and line, transferred to large live cars, and fed daily with generous portions of fresh squid. When a sufficient number of fish for a particular experiment had accumulated, they were placed in smaller indoor tanks, marked so that each individual could be identified, and an initial blood sugar determination was made. Further treatment will be described under separate headings in the experimental section.

Pancreatectomy in the dogfish is a simple matter and merits no description. Partial hypophysectomy is a more complicated operation, however. After immobilization of the animal by cooling in iced seawater (Parker, 1937) the fish was strapped to a V-shaped operating board, and the mouth held open by retractors. An incision about one-half inch in length was made in the oral epithelium, exposing the cartilaginous skull directly over the hypophysis. With a small scalpel, a 3 mm. incision was made in the skull parallel to and slightly posterior to the optic chiasma. Two somewhat longer incisions were then made from the edges of the lateral incision posteriorly and parallel to the sides of the jaws. The flap thus formed was bent caudally until the anterior edge of the neuro-intermediate lobe was visible. For the removal of the anterior lobe alone, the distal part was dissected away from the neuro-intermediate lobe by passing a small curved knife through the connections between the two lobes. The tongue of the anterior lobe was then dissected off the hypothalamus and the entire lobe removed with fine forceps. For the removal of the neuro-intermediate lobe alone, the distal portion of the anterior lobe was dissected free of the neuro-intermediate lobe and deflected slightly. The large neuro-intermediate lobe was then either lifted out, usually in one piece, or removed by suction. For complete hypophysectomy, the procedure was reversed. The tongue of the anterior lobe was removed first, and then the remainder of the pituitary, after severing it from the ventral lobe. Complete hypophysectomy as used here consists of the removal of the anterior and neuro-intermediate lobes. The ventral lobe, of uncertain significance, remained embedded in the cartilage. The sacculus vasculosus was also left intact. When the operation was completed, the cartilagenous flap was allowed to settle in place thus closing the wound, and the oral epithelium sutured. About 4 to 7 minutes are required for hypophysectomy, and about 10 to 15 minutes for pancreatectomy. With assistance both operations can be performed in about 10 minutes.

Blood was obtained from the caudal artery and subsequently analyzed by the macro-precipitation method of Miller and Van Slyke (1936).

EXPERIMENTAL

The Effect of Background on the Resting Blood Sugar

If intermedin is the diabetogenic hormone, it might be expected that fishes subjected to a prolonged adaptation to a black background would have higher blood sugar values than those adapted to a white background, since the former state is produced by a continuous discharge of intermedin whereas the latter state results from an inhibition of the secretion of this hormone.[3] Citation of such a condition in the toad has already been made. Accordingly, dogfishes which had been previously fed for various periods, were brought into the indoor tanks, and an initial

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[3] This version of the physiology of the neuro-intermediate lobe in the dogfish is, however, open to criticism (cf. Abramowitz, 1939*b*) since it is believed by some that the white-adapted state is due to the secretion of a new hypophyseal hormone. However this may turn out, it is generally agreed that during black-adaptation there is an abundance of intermedin secretion, and during white-adaptation, less or probably no circulating intermedin.

Table I

The effect of background on the blood sugar of starved dogfish.
Blood sugar in milligrams per cent.

Initial	Days Starvation after Initial Determinations							Background	
	1	2	3	4	5	6	7		
216	198	168	127	122	117	132	116	Black	*A*
236	140	131	125	141	128	—	110		
Average 226	169	149	126	131	122	132	113		
252	138	130	113	130	98	136	97	White	
219	162	123	101	100	94	106	93		
Average 235	150	126	107	115	96	121	95		
126	130	146	112	107	101	106	124	Black	*B*
116	147	136	—	120	104	113	75		
140	—	156	136	112	126	114	114		
143	150	155	137	116	—	87	87		
Average 131	142	148	128	114	110	105	100		
102	119	108	98	104	111	102	109	White	
112	145	148	148	118	124	110	124		
164	150	149	150	132	134	107	104		
150	149	155	140	117	121	124	135		
Average 132	140	140	134	118	122	111	118		
156	—	135	121	—	103	—	—	Intermediate	
139	—	125	122	—	137	—	—		
121	—	134	132	—	116	—	—		
137	—	125	161	—	120	—	—		
Average 138		129	134		119				
Grand Average 158±11.3	148±5.5	139±3.8	128±4.3	118±3.4	116±3.3	112±4.0	107±4.7		
No. of Animals (16)	(11)	(16)	(15)	(12)	(15)	(11)	(12)		

blood sugar value obtained. The fishes were then placed on white, black, and intermediate backgrounds, starved[4] for the duration of the experiment, and their blood sugar level followed daily.

As the data in Table I clearly show, there is no effect of background on the resting blood sugar level of the dogfish. In one series (Column A, Table I) the black-adapted fish showed higher values than the white-adapted fish, but in a second series (Column B) the order was reversed. There are, however, several significant observations to be pointed out. The initial values in Table I as well as in the following tables show considerable variation. This is mostly due to the extent of previous feeding, for fish that were freshly obtained may show low or high values, whereas animals which were fed for several weeks always show high initial values. This is clearly illustrated in the data (Table I) where the initial readings for well-fed animals (Column A) are nearly twice those of meagerly fed animals (Column B). However, the blood sugar values fall gradually during starvation and reach a constant level after the third day. The level reached is quite independent of the magnitude of the initial values.

Since there was no effect of background on blood sugar level, all of the individual determinations were averaged and the result expressed as grand averages at the foot of Table I. These averages, therefore, represent blood sugar level as a function of the time of starvation and serve as standard values for comparison with results obtained in other experimental series. It can be seen that after the second day of starvation the standard deviation of the averages assumes a low constant value, while the variation of the initial determinations is considerable, as seen from the large standard deviation. Consequently, the results for animals on which operations were performed cannot be calculated as percentages of the initial blood sugar values before operation because the initial values are not constant. The only valid comparisons are those made in relation to the standard values on the same day of starvation.

The Effect of Hypophysectomy

Orias (1932) concluded that complete hypophysectomy did not affect the blood sugar of the dogfish, since the average value for 8 fish on the second day following hypophysectomy was no different from that previous to the operation. Orias' observations are undoubtedly correct inasmuch as our data (Table II) likewise show no difference between the hypophysectomized and control groups (Table I) on the second day of starvation. However, removal of the pituitary leads to a marked hypoglycemia (89–88 mg. per cent) at the fourth day of starvation. There is considerable variation, however.

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[4] In all of the experiments, the animals were starved throughout the course of an experiment.

The effect of partial hypophysectomy was determined by removal of only the anterior lobe in 14 animals, and of only the neuro-intermediate lobe in 11 animals. Both sets of animals were followed for a week. The effect of the removal of the anterior lobe (Table III) is very similar to that of complete hypophysectomy. The level reached (96–94 mg. per cent) is not as low as that following complete hypophysectomy, but there is less variation. Removal of the neuro-intermediate lobe (Table IV), however, does not lead to hypoglycemia, and the average values for this series are remarkably close to those of the control series (Table I). The results of the three series of operations as compared with the control series are summarized in Table V.

TABLE II

The effect of the removal of the pituitary on the blood sugar of starved dogfish. Blood sugar in milligrams per cent.

Initial	Days starvation following operation						
	1	2	3	4	6	9	11
108	—	101	—	72	53		
190	—	99	—	97	89	90	82
216	—	201	—	161	158	129	121
201	—	115	—	98	110	88	
290	—	78	—	63	82		
117	—	270	—	126			
228	—	190	—				
151	102	87	83	70	93		
132	131	117	109	114	75		
177	—	112	—	101	103		
121	—	104	—	35			
112	—	—	—	42	30		
Ave. 170	—	134±17.2	—	89±10.8	88±11.3		
No. of Animals	—	(11)	—	(11)	(9)		

It can be concluded that the removal of the anterior lobe produces the same effect as that seen after complete hypophysectomy and that the ablation of the neuro-intermediate lobe has no effect on blood sugar. Furthermore, the hypoglycemia, which follows the removal of the anterior lobe, occurs in spite of a continuous secretion of intermedin (Abramowitz, 1939*b*), and on the other hand, hypoglycemia does not occur after ablation of the neuro-intermediate lobe which deprives the dogfish entirely of intermedin.

TABLE III

The effect of the removal of the anterior lobe of the pituitary on the blood sugar of starved dogfish. Blood sugar in milligrams per cent.

Initial	Days starvation following operation				
	1	2	3	4	6
124	—	106	—	126	85
168					
118	—	107	—	89	89
240	—	116	—	105	110
137	—	165	—	88	—
111	—	114	—	110	121
155	—	103	—	90	115
260					
130	—	88	—	64	32
163	168	112	131	117	113
132	132	113	100	83	
126	—	122	—	93	110
112	—	118	—	94	71
107	—	118	—	90	90
Ave. 148	—	115±5	—	96±4.5	94±8
No. of Animals	—	(12)	—	(12)	(10)

TABLE IV

The effect of the removal of the intermediate lobe of the pituitary on the blood sugar of starved dogfish. Blood sugar in milligrams per cent.

Initial	Days starvation following operation					
	1	2	3	4	5	7
117	—	193				
154	—	166	110	143		
125	—	109	107	98	115	107
161	—	164	128	117	110	187
122	—	163	111	104	124	141
128	—	130	109	109	—	
134	186	111	113	106	112	
102	114	113	122	110	144	
128	—	124	112	—	114	
132	—	116	89	—	95	
130	—	145	114	—	119	
Ave. 130		139±8.2	112±3.0	112±5.2	117±4.6	
No. of Animals		(11)	(10)	(7)	(8)	

The experiments just described furnish good evidence that the presence or absence of intermedin is without effect on the resting blood sugar level. The most crucial test of the identity of the diabetogenic and chromatophorotropic hormones of the pituitary, however, is one performed on the depancreatized animal. If the continued presence of intermedin in the body is not accompanied by an aggravation of experimental diabetes, and if its complete absence is not accompanied by an amelioration of diabetes, there can be little doubt that intermedin is not the diabetogenic factor. Therefore, the effects of background and of partial hypophysectomy on the blood sugar level of depancreatized dogfish were determined. The results of this series of experiments follow.

TABLE V

Summary table showing the effects of partial and complete hypophysectomy on the resting blood sugar level of starved dogfish.

Operation	Initial value	Blood Sugar in Milligrams Per Cent			
		Days following operation			
		2	4	5	6
Controls	158	139	118		112
Complete hypophysectomy	170	134	89		88
Anterior lobe removed	148	115	96		94
Intermediate lobe removed	130	139	112	117	

Pancreatic Diabetes

Twenty-five fish, fed for various periods of time, were depancreatized after an initial determination had been obtained. Nine were placed in white and sixteen in black tanks. Both sets were starved and the blood sugar values determined daily. The results (Table VI) show that the severity of diabetes is not influenced by the relative amounts of circulating intermedin. In some instances, the black-adapted group showed a slightly more intense diabetes, and in other instances, the reverse was true. All the data were therefore averaged, and the values, given at the foot of Table VI, indicate merely the blood sugar level of depancreatized animals on various days of starvation. The depancreatized animals show values roughly three times those of the control series.

An interesting point with regard to the effects of pancreatectomy in the dogfish is the fact that the severity of the resulting diabetes is independent of the blood sugar level prior to the operation. Initial values around 200 mg. per cent indicate fish which had been fed for several weeks prior to operation, yet the resulting diabetes following pancreatectomy is approximately the same as that of poorly fed animals. For example, the initial value of 98 mg. per cent (poorly fed fish) rose to 422, 473 and 500 mg. per cent, a level which is as high as that found in the well-fed individuals. This illustrates again that the results cannot be calculated in terms of the initial values. Secondly, there appears to be no uniformity among the individual fish during the course of their diabetic condition. Some of the animals become progressively more diabetic, some maintain a constant level, and some tend to show decreasing hyperglycemias after the first twenty-four hours.

TABLE VI

Pancreatic diabetes in dogfish starved after initial readings.
Blood sugar in milligrams per cent.

Initial	Days Starvation after Pancreatectomy									
	1	2	3	4	5	6	7	8		Background
193	235	208	255	195						White
175	275	210	270	242	191	175				
212	319	291	346	442	508	514	583	496	410	
156	272	172	175	242						
215	371	381	512	584						
269	377	294								
231	282	245	226							
284	380	342	593							
232	452	330	392	393						
Average 213	320	275	347	350						
149	—	—	250							Black
198	—	—	216							
209	—	—	150							
234	391	—	392							
217	356	388	—							
98	422	473	500	445						
134	356	418								
135	562	258								
125	—	—	130							
236	—	—	432							
197	—	—	280							
146	—	—	400							
160	—	—	350							
198	—	—	526							
Average 174	417	384	330							
[120	94	44]								
[94	29	30]								
Grand Average 191	361±22	308±24.2	337±30	363±50						
No. of Animals (23)	(14)	(13)	(19)	(7)						

Inspection of the values listed in Table VI reveals a considerable individual variation. This is somewhat contrary to the results obtained by Orias whose nine depancreatized animals had values between 350 and 431 mg. per cent with an average of 402 mg. per cent at 48 hours fol-

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lowing operation. Our results can be arbitrarily classified into four main groups: (1) severe diabetes, (2) mild diabetes, (3) no diabetes, and (4) hypoglycemia. The first group represents values above 300 mg. per cent, the second between 200 and 300 mg. per cent, the third between 100 and 200 mg. per cent, and the fourth, values below 100 mg. per cent which is below that of a normal animal. Such a classification is, of

TABLE VII

Effects of complete and partial hypophysectomy on pancreatic diabetes. Blood sugar in milligrams per cent.

Pancreas + Pituitary removed	Pancreas + Anterior lobe removed	Pancreas + Intermediate lobe removed
100	[96	[96
110	79	28]
117	43	
133	88]	
176		
173		175
134		182
	159	120
	146	126
	121	196
368	120	164
350	114	
370	143	
311	119	
425		800^+
437		391
		385
	335	335
	433	484
212	382	548
233	333	682
289	462	307
213		478
292		319
282		445
293	285	689
273	284	392
204	228	
247	206	
294	202	
212	298	284
224	233	297
	219	254
		226
		211
Ave. 249±18	241±23	354±36
No. of Animals (26)	(20)	(24)

course, quite rough, but it serves to bring out a significant comparison with results obtained in the next section where both pancreas and pituitary were removed. The percentages of the animals falling into the various groups are shown in Table VIII. Most of the depancreatized animals (56 per cent) become severely diabetic, 24–28 per cent are only mildly diabetic, and 8–12 per cent do not develop hyperglycemic levels at all. That a small percentage of animals does not become diabetic seems to be a constant factor, for a similar percentage has been found in all cases involving removal of the pancreas. Two of the twenty-five animals, or 8 per cent, developed hypoglycemia. These two animals were not included in the calculations of the averages in Table VI, since they are obviously anomalous. This, too, appears to be a constant factor for it has recurred in the other series of operations in about the same percentage of cases.

TABLE VIII

Summary table showing distribution of results among arbitrary classes of diabetes. Figures indicate percentage of the animals comprising the various types.

Operation	Hypoglycemia	No Diabetes	Mild Diabetes	Severe Diabetes
Range of blood sugar, Mg. %	<100	100–200	200–300	>300
Pancreas alone removed	8%	8–12%	24–28%	56%
Pituitary + pancreas removed	0–3.85%	23.2–26.8%	50%	23.2%
Anterior lobe + pancreas removed	16.6%	29.4%	33.6%	20.4%
Intermediate lobe + pancreas removed	7.7%	23.1%	19.2%	50.0%

A few animals were followed immediately after pancreatectomy to determine how soon hyperglycemia appeared. The blood sugar rose shortly after operation and reached peak levels within nine to twelve hours.

The Effect of Complete and Partial Hypophysectomy on Experimental Diabetes

Seventy-six animals were depancreatized and hypophysectomized simultaneously. In 26 animals, the pancreas and the whole pituitary were removed; in 26 the pancreas and only the neuro-intermediate lobe were removed; and in 24 the pancreas and only the anterior lobe were removed. Blood sugar analyses were made daily, but due to high mortality, only the determinations on the first day following operation were listed in Table VII. An individual variation in the severity of diabetes, such as that described for pancreatectomy, was encountered, and the percentage of cases showing the various types of diabetes is given, for each of the 3 series of operations, in Table VIII.

Complete hypophysectomy tends to reduce the severity of experimental diabetes. As shown in Table VII, the average blood sugar level is 249 mg. per cent, which is somewhat lower than the average of 9 animals (281 mg. per cent) found by Orias. The greatest number of animals (50 per cent) show a mild type of diabetes, and 23 per cent show either no diabetes or a severe diabetes. None of the animals was hypoglycemic at the first day, but 2 animals showing the lowest readings (100 and 110 mg. per cent) became hypoglycemic on the third and fourth days.

The average value of 20 animals from which both the anterior lobe and the pancreas were removed was 241 mg. per cent, which is practically the same level as that found in completely hypophysectomized-depancreatized animals. Sixteen per cent showed hypoglycemia and was not included in the calculation of the average for this group. Twenty-nine per cent showed no diabetes and 20 per cent showed a severe type. On the whole, the results of this group are similar to those obtained when the pancreas and the entire pituitary were removed.

The results obtained when only the neuro-intermediate lobe and pancreas were removed are quite different from those of the other two groups of animals in this series of operations but similar to those obtained when only the pancreas was removed. The average value of this group (excluding the 8 per cent showing hypoglycemic levels) was 354 mg. per cent. Most of the animals (50 per cent) showed severe diabetes, 19 per cent showed mild diabetes, and 23 per cent were not diabetic. One of the animals showed an extremely high level of 800 mg. per cent, which is probably an anomalous value. Excluding this value, the average of this group would be somewhat lower (334 mg. per cent), which is still a severely diabetic level.

Examination of the summary table (Table VIII) leaves little doubt that complete hypophysectomy alleviates pancreatic diabetes. Anterior lobe removal accomplishes the same result but the removal of the neuro-intermediate lobe has little effect on the severity of experimental diabetes. These results are consistent with those of the previous experimental series, and lead to the conclusion that the diabetogenic hormone in the dogfish is produced by the anterior lobe of the pituitary, and that this hormone is not identical with intermedin.

SUMMARY

The resting blood sugar level and the severity of pancreatic diabetes are not influenced by fluctuations in intermedin secretion in the dogfish.

Complete hypophysectomy is followed by the appearance of hypoglycemia on the fourth day following the operation, a response which is also produced by the removal of the anterior lobe alone. Removal of the neuro-intermediate lobe does not affect the resting blood sugar level. Complete hypophysectomy ameliorates the severity of pancreatic diabetes, as does the removal of the anterior lobe alone. The removal of the neuro-intermediate lobe alone does not influence pancreatic diabetes. These results show that intermedin and the diabetogenic hormones of the pituitary of the dogfish are not identical.

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A NEW METHOD OF PRODUCING TWINS, TRIPLETS AND QUADRUPLETS IN ARBACIA PUNCTULATA, AND THEIR DEVELOPMENT

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Method

The simplest method of obtaining twins experimentally is from the first two blastomeres of a developing egg. In the early classical experiments of Driesch (1891, 1892), the isolated blastomeres of the sea-urchin egg were obtained by shaking. Later, he (1900) and most investigators since, have made use of Herbst's (1900) discovery that in Ca-free sea water, the ectoplasmic layer which binds the blastomeres together is dissolved and the blastomeres fall apart spontaneously or with slight shaking. Dilute sea water, heat, and the lack of certain ions (other than Ca) in the sea water also cause the blastomeres to separate (Driesch, 1892, 1906; Loeb, 1909). Some recent investigators have used free-hand cutting with glass needles, and others have used this method in combination with Ca-free sea water. These methods are tedious and the paired blastomeres must be isolated at an early stage; moreover, as pointed out by others, the procedure is injurious to the eggs, so that many of the separated blastomeres do not reach the blastula stage, and comparatively few the pluteus; it is therefore difficult to evaluate the results.

A new and much more efficient method of obtaining large quantities of twins and quadruplets from isolated blastomeres of the *Arbacia* egg was found in the course of some other experiments. No extensive work has been done, but the method is so simple and the general results so striking that it seems worth while to put them on record. The procedure is as follows. The eggs are fertilized, and the fertilization membranes removed by shaking two minutes later. The eggs are then allowed to develop in ordinary sea water till just after first cleavage (53 minutes after fertilization at 23° C.). Then the eggs are placed in hypertonic sea water, made either by boiling sea water to half its volume, or by adding 30 grams of NaCl per liter of sea water (same solution as used by me (1936) for parthenogenesis). The eggs are left in this solution 5–10 minutes, then returned to sea water. The first two blastomeres are nicely separated and develop independently forming a pair of twins, joined by a thin cord or film. They swim at first in pairs, and can be easily isolated at this stage and their development followed; they soon become entirely independent of each other. By this method, large quantities can be obtained, and the pairs are isolated at a much later stage than by previous methods. In this way, eggs which have been injured by shaking or by the changed concentration of the sea water are eliminated, and since only very healthy-looking, active swimmers need be chosen, their fate seems a good criterion of the final development of isolated blastomeres. The older idea of the totipotency of the blastomeres of the echinoderm egg has been questioned by some more recent investigators. Of course, one might claim that all blastomeres lacking certain materials or cleaved in some particular plane died before reaching the blastula stage, but this seems to me improbable.

Twins

The production of the twins is apparently due to the effect of the hypertonic sea water on the ectoplasmic layer which binds the cells together. The hypertonic sea water causes the ectoplasmic layer to become very thick (Cf. Photograph 1 with 2, the control), and this is true for the eggs at any stage, and whether with or without fertilization membranes. At the time of cleavage, the thickened layer forms a heavy coat around each blastomere (Photograph 1). When the eggs are returned from the hypertonic sea water to ordinary sea water, the ectoplasmic layer swells and spreads out, becoming thin and gelatinous, and the two blastomeres are often widely separated with only a thin film between (Photograph 3). Any protoplasmic connection is soon broken. Apparently then, blastomeres can be separated both by a thickening of the ectoplasmic layer caused by hypertonic sea water, and by a dissolution of the layer caused by absence of calcium or by hypotonic sea water. It may be, however, that in the present case, the change from the hypertonic solution to the isotonic sea water is responsible for the effect rather than the hypertonic solution itself. If the fertilization membranes have not been removed, the two blastomeres do not develop separately, but apparently are so pressed together that they develop as one embryo.

Each blastomere divides into two equal cells at the same time that the whole egg divides into four (Photographs 4, 5), and into four equal cells when the whole egg divides into eight (Photograph 6). No critical work has been done on the micromeres, but in some cases two were present after the next cleavage, and in some cases they were not observed. By subsequent cleavages, each original blastomere gives rise to a spherical blastula, quite normal in appearance, but of half the normal volume and with half the normal number of cells. The blastula is not the open type like the half blastula of *Parechinus*, but is a closed sphere like that of *Sphaerechinus* (Driesch). The half blastulae become free-swimming at the same time as the whole blastulae (Photographs 7–9). The gelatinous material remaining from the ectoplasmic layer still holds the pairs together even after they become actively free-swimming (Photograph 9), and it is at this time that one isolates the twins to follow their history.

An hour or so after becoming free-swimming, the twins separate and swim independently. The later development is slower than that of the whole blastula; the pluteus stage is reached on the third day instead of the second. In some cases in which individual pairs were isolated, both blastulae developed into plutei, perfect in structure, but smaller than normal (Photograph 10, *A, B*). In some cases, one developed into a perfect pluteus, the other was underdeveloped or abnormal (Photograph 11), and in some cases both developed abnormally or remained in an arrested state of development (Photograph 12).[1] Those which are designated as underdeveloped and abnormal were large blastulae ("Dauernblastulae"?) and gastrulae which did not subsequently become plutei, and plutei with abnormal skeleton or arms. When isolated in lots of 5 or 10 pairs of twins, in some batches few became perfect plutei (Photograph

PLATE I

Twins, Early Development

The photographs were taken with a Leica camera, of living eggs and embryos of *Arbacia punctulata,* and are not retouched; the swimmers were narcotized with chloretone. Most of the photographs were taken with a water immersion (× 40) lens and a 10 × ocular and are magnified about 250 × as presented. Photographs 10–13 and 30 were taken with a 16 mm. (× 10) lens and a 20 × ocular and are magnified about 125 ×; Photograph 7, same optical system but now magnified 250 ×. Photographs 14–16 and 24, 25, 38, 41 were taken with a 16 mm. (× 10) lens and a 10 × ocular and are magnified about 60 × as presented.

PHOTOGRAPH 1. Eggs in hypertonic sea water at the time of first cleavage. Note thickened ectoplasmic layer.

PHOTOGRAPH 2. Control; normal eggs in sea water with normal ectoplasmic layer.

PHOTOGRAPH 3. Eggs 7 minutes after return to sea water from hypertonic sea water. Two blastomeres separated with thin film between. One and one-quarter hours after fertilization.

PHOTOGRAPHS 4, 5. Each blastomere 2-cell. One and three-quarters hours after fertilization.

PHOTOGRAPH 6. Each blastomere 4-cell. Two hours after fertilization.

PHOTOGRAPH 7. Each blastomere 16-cell. Three hours after fertilization.

PHOTOGRAPH 8. Each blastomere an early blastula. A whole blastula to right. Four hours after fertilization.

PHOTOGRAPH 9. Twin blastulae just before becoming free-swimming. Five and one-half hours after fertilization.

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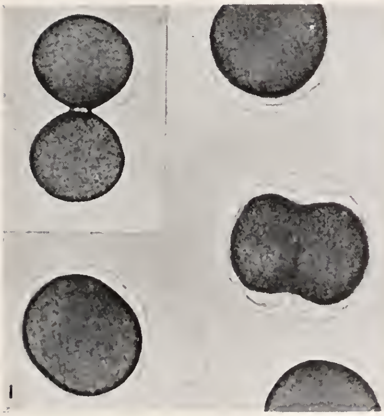
[1] These three classes occurred in approximately equal numbers in my experiments.



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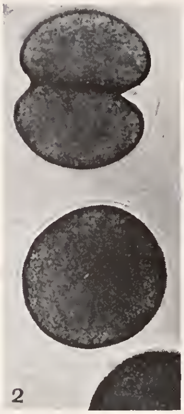

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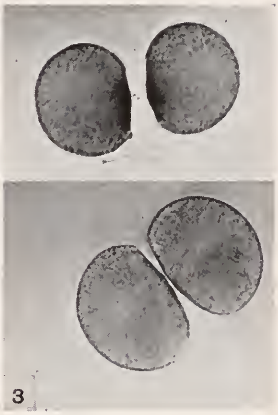

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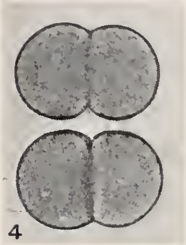

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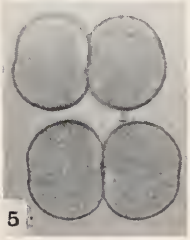

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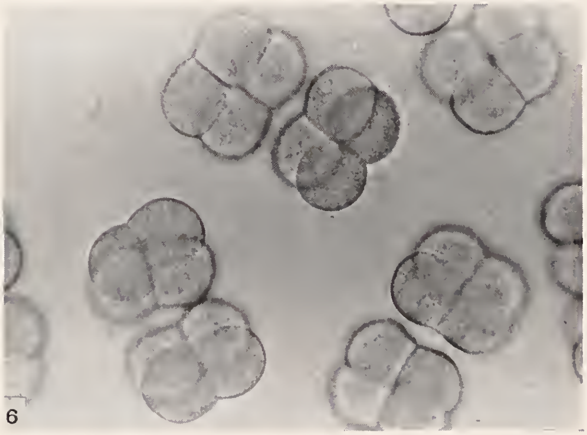

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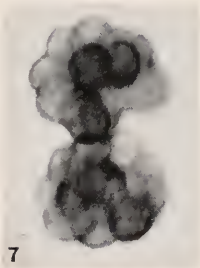

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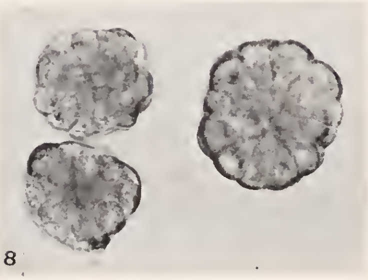

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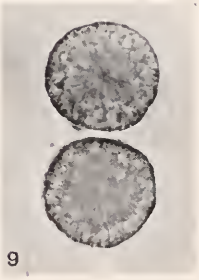

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PLATE I

15), in other batches, many. In one batch all of the twenty (from 10 pairs) were perfect plutei except one which was a well-formed gastrula (Photograph 14). In such lots, one embryo may reach the pluteus stage a day before the others. As a control to the twins, some whole blastulae from the experimental lots were isolated at the same time in pairs and in lots of 20, and though they looked in fine condition, they gave rise to the same sort of abnormal forms as the twins but in a smaller proportion. A comparison of Photograph 13 with 11 and of Photograph 16 with 14 will show the similarity in the development of the whole and half blastulae. Even when normal blastulae are isolated in countable lots from *normal* cultures, usually one or two do not develop perfectly normally.

Since the results are so variable in different lots of the twins and since similar abnormalities occur also among the whole blastulae, it seems probable that the abnormal development is due to the experimental conditions (most likely to the moist chamber) and to innate peculiarities of the eggs, rather than to any lack of special materials separated off by cleavage planes. This variability in different experimental lots must be taken into account by investigators in this and related fields who, owing to the difficulties of operation, necessarily use small numbers of eggs, and often do not keep adequate controls. There is no evidence whatever of any proportion of the twins lacking gut-forming or skeleton-forming material and therefore remaining as blastulae. Permanent blastulae ("Dauernblastulae") do occur, but they also occur in large numbers in certain supposedly normal lots of developing whole eggs which have not been "ectodermised." And there is no evidence that the two twins are complementary so that the first two blastomeres represent the right and left sides of the embryo. Occasional one-armed plutei do

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PLATE II

Twins, Later Development

PHOTOGRAPH 10, *A*, *B*. Two twin plutei from the same egg, isolated as blastulae. Both perfect. Two days old.

PHOTOGRAPH 11. One twin perfect, one underdeveloped, from the same egg. Two days old.

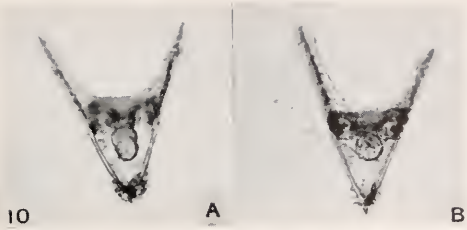
PHOTOGRAPH 12. Both twins underdeveloped or abnormal. Two days old.

PHOTOGRAPH 13. One perfect pluteus, one abnormal; from two whole blastulae, isolated from same dish as the above sets of twins. One day old. Cf. with Photograph 11.

PHOTOGRAPH 14. Plutei from 10 pairs of twin blastulae. All perfect except one, a good gastrula. Three days old.

PHOTOGRAPH 15. Another set of 5 pairs from another culture. One perfect pluteus, one nearly perfect, 6 gastrulae with skeleton, 2 blastulae without skeleton. Three days old.

PHOTOGRAPH 16. A lot of 21 whole embryos isolated at the blastula stage from experimental culture (as a control to twins). Note several imperfect or underdeveloped. Two days old. Cf. with Photographs 14, 15.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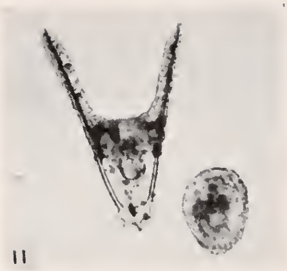
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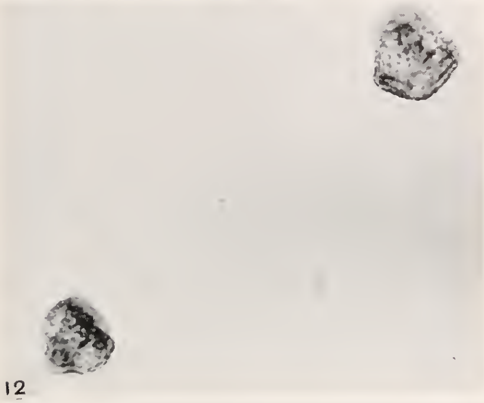
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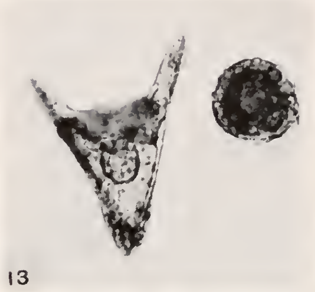
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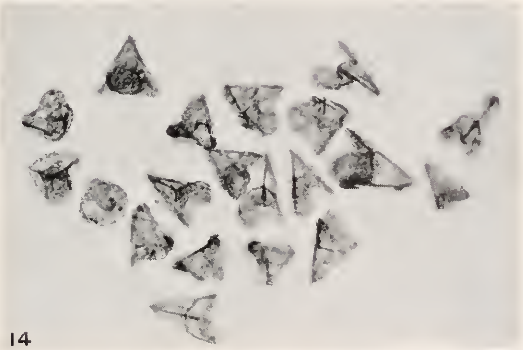
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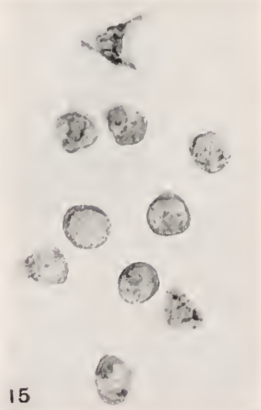
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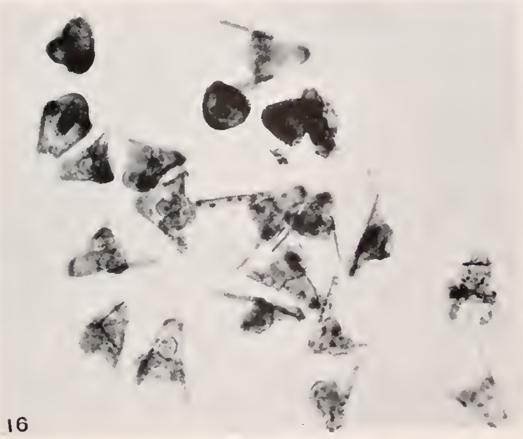
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PLATE II

occur, but these have been found also in very different experiments. Nor does there seem to be any dorsal or ventral predominance in either of the developing blastomeres. These conclusions as to the early organization of the echinoderm egg, and especially of the *Arbacia* egg, are in general opposed to those of Plough (1927, 1929) and Hörstadius 1928, 1936, 1939), and are more in line with the original ideas of Driesch and those expressed by Wilson in " The Cell," and the more recent ones of von Ubisch (1936, 1938). It may be seen from Photographs 10, *A*, *B* and 14 that the first two blastomeres can and under optimum conditions, probably do develop into normal small plutei. The first cleavage plane must either divide the egg into two exactly similar parts, or else each of the first two blastomeres must be able to regenerate any material necessary for a normal pluteus which has been segregated from its mate by the first cleavage plane. It might be pointed out that the eggs of different species of sea urchin are quite different, as shown by their appearance and by their stratification with centrifugal force. Some may be more highly organized than others, and materials may be more localized. One has only to consider the pigment band in the *Paracentrotus* egg, which is not found in other species, and varies greatly in intensity in that species in different localities, and even in the same locality. This may be visible evidence of a greater localization of materials in this egg than in the *Arbacia* egg. The first two blastomeres of *Arbacia punctulata* are certainly totipotent, whatever they may be in other sea urchins.

Quadruplets

If the *Arbacia* eggs from which fertilization membranes have been removed are placed in the hypertonic sea water just after second cleav-

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PLATE III

Quadruplets

PHOTOGRAPH 17. Eggs in hypertonic sea water just after second cleavage. Note thickened ectoplasmic layer.

PHOTOGRAPH 18. Eggs 13 minutes after return to sea water from hypertonic sea water. Four blastomeres separated with film between. One and three-quarters hours after fertilization.

PHOTOGRAPH 19. Each blastomere 2-cell. Two hours after fertilization.

PHOTOGRAPH 20. Asynchronous first cleavage of four separated blastomeres. Two hours after fertilization.

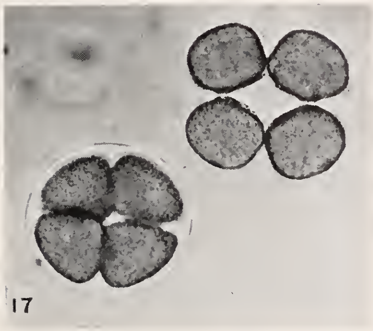
PHOTOGRAPH 21. Each blastomere 4-cell. Two and three-quarters hours after fertilization.

PHOTOGRAPH 22. Quadruplets in later cleavage. A pair of twins above, and two whole eggs below. Three and one-quarter hours after fertilization.

PHOTOGRAPH 23. Quadruplets just before becoming free-swimming. Note film still holding them together. Five hours after fertilization.

PHOTOGRAPH 24. Quadruplets from same egg, all perfect plutei. Two days old.

PHOTOGRAPH 25. Two sets of quadruplets, all imperfect plutei with skeleton. Three days old.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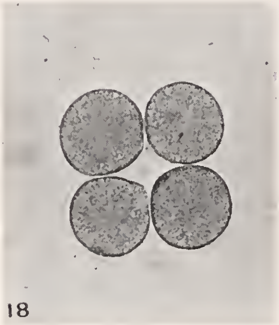
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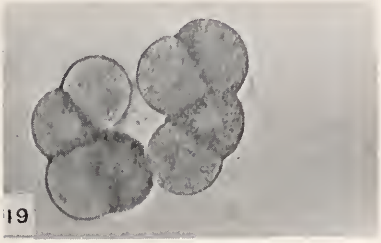
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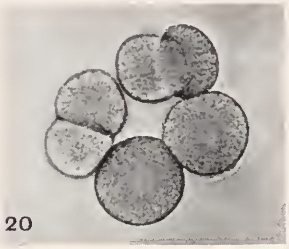
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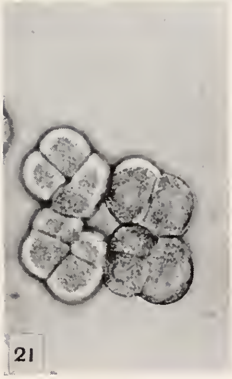
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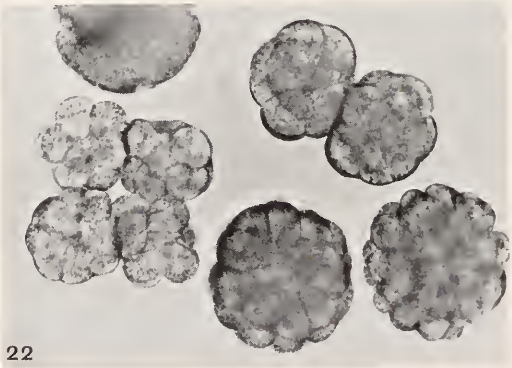
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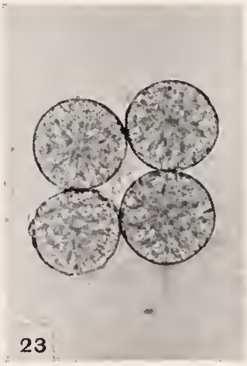
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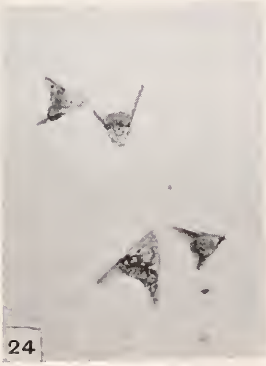
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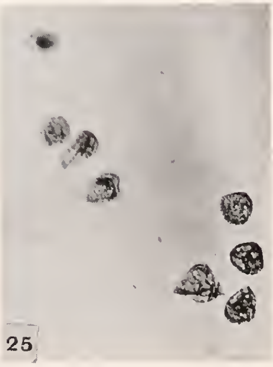
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PLATE III

age, the ectoplasmic layer between the four cells becomes thickened (Photograph 17). When, after 5–10 minutes, they are returned to ordinary sea water, the four cells become separated but linked together by a film of gelatinous material (Photograph 18). Each blastomere cleaves at the same time as the whole egg, though one cell may be in advance of the others (Photographs 19, 20). Each blastomere passes through subsequent cleavages independently, until four small (closed) blastulae are formed (Photographs 21, 22, 23). These swim together at first in quartets, and it is at this stage that they are isolated; they soon break apart and become independent swimmers. The quadruplets cleave at the same time and become free-swimming at the same time as the twins and whole eggs. Each one has therefore only one quarter the normal number of cells. Some of the sets of four blastomeres give rise to plutei all absolutely normal except in size (Photograph 24). There is some individual variation in size but this is true also of individuals coming from whole eggs. Some quartets give rise to plutei somewhat abnormal or underdeveloped but usually with skeletons (Photograph 25). The plutei from the quarter blastulae are formed more slowly than from whole blastulae.

The development of the quadruplets gives no indication that any one of them lacks any particular material segregated by the first or second cleavage planes. Nor is there any evidence of any dominance of a right or left side, or of a dorsal or ventral part. Each of the quadruplets is in general like its mates, but some lots develop much better than others, owing probably to experimental conditions or to innate differences in vitality. The second cleavage plane, as well as the first, either divides the egg into exactly similar parts, or else each blastomere regenerates any material segregated into the others by the cleavage planes.

The quartets would serve as excellent material for a more critical study since it is quite possible to tell the exact relation of each member of the quartet to the first and second cleavage planes; the second plane is

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PLATE IV

Triplets and Fused Twins

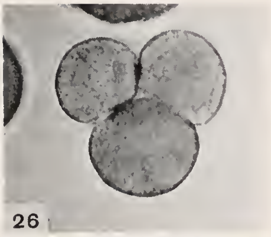
PHOTOGRAPHS 26, 27. Beginning of triplets. Two blastomeres at 4-cell stage remain separate, and other two remain together or fuse. One and three-quarters hours after fertilization.

PHOTOGRAPH 28. Fusion of two of quadruplets at a later stage, forming triplets. Three and one-quarter hours after fertilization.

PHOTOGRAPH 29. Triplets in blastula stage. Whole blastula to left. Four and one-quarter hours after fertilization.

PHOTOGRAPH 30. Triplets. One large perfect pluteus, one small pluteus slightly imperfect, one small underdeveloped pluteus with skeleton. Two days old.

PHOTOGRAPH 31. Blastulae from partially fused twins (2 center ones). A pair of twins to left, and a whole blastula to right. Four hours after fertilization.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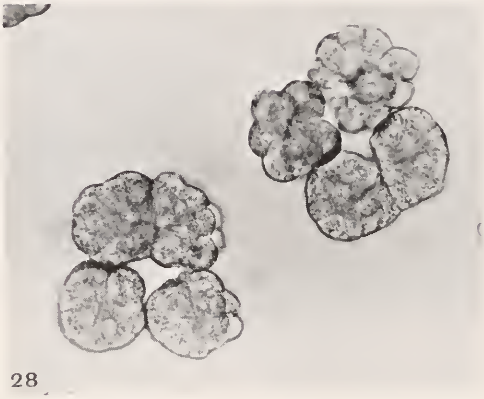
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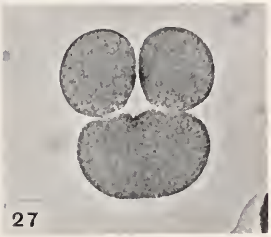
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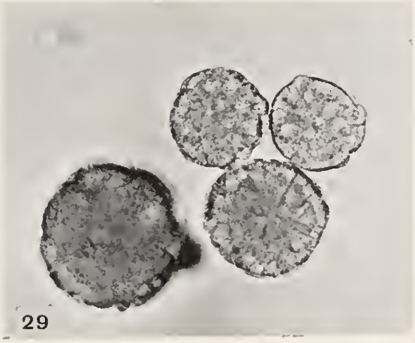
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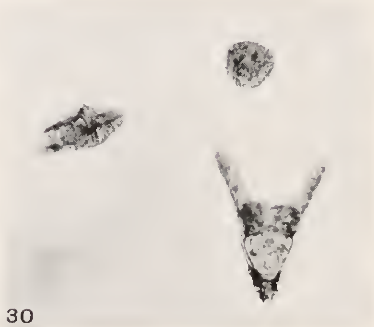
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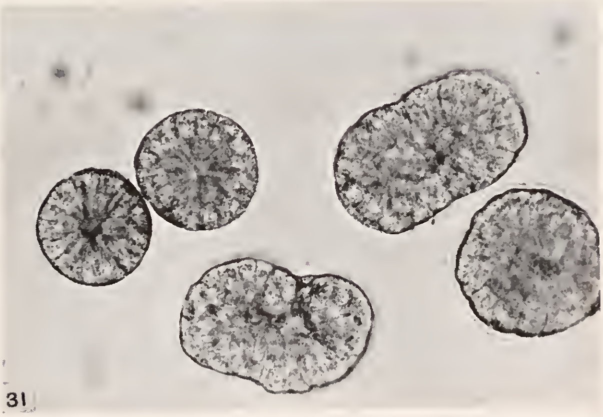
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PLATE IV

longer than the first (Photograph 17). This is due to the fact that the pair of cells made by the first cleavage plane is more widely separated at the four-cell stage than the pair made by the second cleavage plane. It is probably on this account that twins as well as quadruplets occur in quantity just after the second cleavage (after treatment with hypertonic sea water). There was no difference in development of these twins, after treatment at the four-cell stage (Photograph 22), and those obtained after treatment at the two-cell stage.

Attempts to separate the eight blastomeres at the next cleavage were unsuccessful; the cells always came together again and developed as a whole. But this method might be used successfully for the eight-cell stage in combination with some other treatment.

Triplets

Triplets are obtained from the quadruplet sets in which only two of the cells remain separate, and the other two develop as a whole (Photographs 26, 27); or two of the four which have started to develop independently, later on become fused while the other two remain distinct (Photograph 28). The triplets, consisting of one large and two small parts, develop into free-swimming blastulae, and they can be isolated at this stage (Photograph 29). These develop into one large pluteus (corresponding to one twin) and two smaller ones (corresponding to two

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PLATE V

Twins Obtained by Centrifugal Force

PHOTOGRAPH 32. Egg after having been centrifuged 40 minutes after fertilization at about 10,000 × g for 6 minutes. Note the ectoplasmic layer lying as a crescent in the broken fertilization membrane.

PHOTOGRAPH 33. Two blastomeres after separation by centrifugal force. Centrifuged 1 hour after fertilization, photographed immediately after removal from centrifuge.

PHOTOGRAPH 34. Same pair, each blastomere 2-cell. Two hours after fertilization.

PHOTOGRAPH 35. Same pair, each blastomere 4-cell. Three hours after fertilization.

PHOTOGRAPH 36. Same pair, each blastomere 8-cell. Three and one-quarter hours after fertilization.

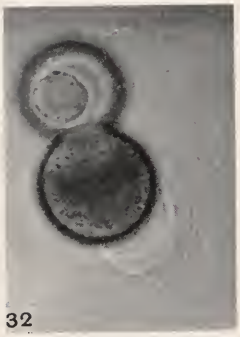
PHOTOGRAPH 37. Twin blastulae just before becoming free-swimming. Six hours after fertilization.

PHOTOGRAPH 38. Two twin plutei from the same egg, isolated as blastulae. Both perfect. Three days old.

PHOTOGRAPH 39. Twin blastulae inside fertilization membrane. Six hours after fertilization.

PHOTOGRAPH 40. Blastula from partially fused twins. Six hours after fertilization.

PHOTOGRAPH 41. Eggs in two-cell stage centrifuged on the centrifuge microscope, photographed while rotating.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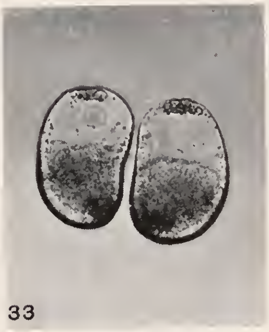
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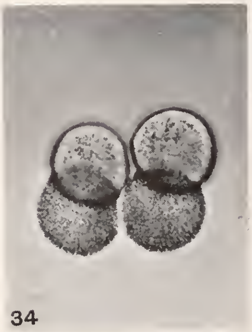
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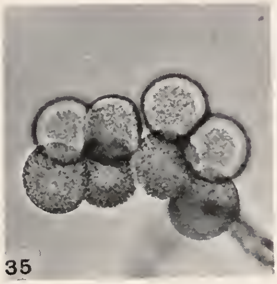
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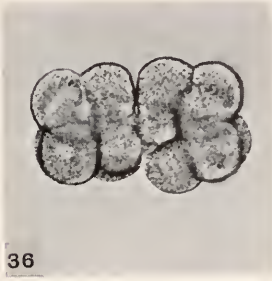
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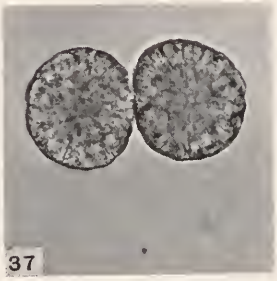
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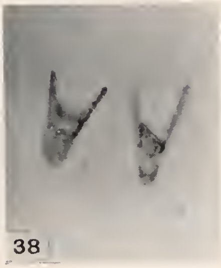
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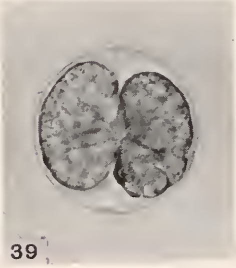
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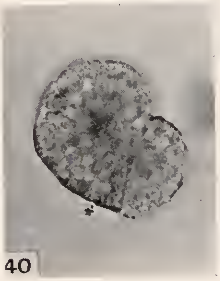
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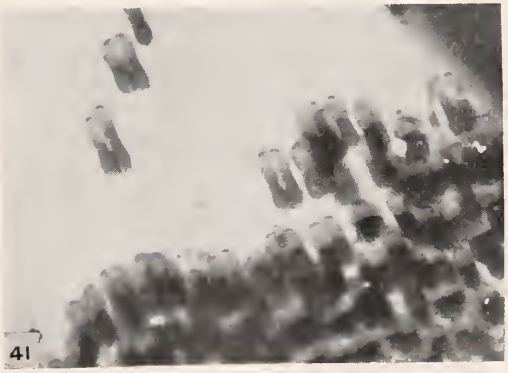
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PLATE V

quadruplets) all of which may be more or less perfect, varying in different lots (Photograph 30).

Fused Twins

After the first two blastomeres have been separated and have started to develop independently, they may again fuse. All degrees of fusion occur, and these fused forms become free-swimming. These may be seen in Photograph 31 together with a pair of twins and a whole blastula. Their development has not been followed. Many of the more thoroughly fused twins are probably indistinguishable from normal whole blastulae, and this may partly explain the unexpectedly large number of imperfect forms developing in the controls to the twins (Photograph 16).

Twins Obtained by Centrifugal Force

Another entirely different method of obtaining twins was discovered several years ago for *Parechinus microtuberculatus* (E. B. Harvey, 1935). It is by centrifugal force, and this method is concerned also with the ectoplasmic layer. This layer can be centrifuged off from the surface of the egg at any stage of development (E. B. Harvey, 1934). When centrifuged off just after first cleavage, the two blastomeres are separated and may develop independently. The same phenomenon can be observed also in the *Arbacia* egg. The force used was about 10,000 × g. for 6 minutes. In Photograph 32, the ectoplasmic layer is seen as a crescent inside the broken fertilization membrane. Photograph 41 shows the eggs in the two-cell stage as they appear during centrifugation on the centrifuge-microscope. In Photograph 33, the two blastomeres are separated, and the independent development of the two blastomeres into two perfect plutei is shown in Photographs 34 to 38. The blastomeres remain attached until after the blastulae have become free-swimming and they can be isolated at this stage. In these cases the fertilization membrane had been removed before centrifuging, by shaking. The two blastomeres may also develop independently inside the fertilization membrane (Photograph 39). They may also fuse again after having been separated (Photograph 40). This method of obtaining twins is neither as simple nor as efficient as the one described in the earlier part of the paper.

Summary

1. Twins, triplets, and quadruplets of *Arbacia punctulata* are obtained by treating the eggs just after first and second cleavage with a hypertonic salt solution, which thickens the ectoplasmic layer; the blasto-

meres separate when returned to sea water. The blastomeres develop independently but attached to each other until they become free-swimming blastulae, when they can be isolated in pairs or quartets.

2. Twins, triplets and quadruplets from a single egg may all develop into perfect dwarf plutei.

3. There is no indication of any differences among the twins, triplets, and quadruplets of a set (i.e. from one egg) caused by the segregation of any special organ-forming materials by the first two cleavage planes, nor by a separation into a right and left half, or into an anterior and posterior part.

4. Differences and abnormalities in the development of twins, triplets, and quadruplets are probably caused by experimental conditions or by differences in vitality of the original egg, since abnormalities of the same sort occur among the controls, and since some lots develop much better than others.

5. Twins may also be obtained by centrifuging the eggs just after first cleavage. The ectoplasmic layer is centrifuged off, and the two blastomeres develop independently but attached to each other until they become free-swimming blastulae. Both twins may form perfect dwarf plutei.

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PRODUCTION OF EYE COLOR HORMONE BY THE EYES OF DROSOPHILA MELANOGASTER[1]

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INTRODUCTION

Two diffusible substances, known as v^+ and cn^+ hormones, are involved in the differentiation of certain eye colors in *Drosophila melanogaster* (Ephrussi, 1938). Evidence that eye tissues produce the hormones was presented by Beadle and Ephrussi (1936) and by Ephrussi and Chevais (1938).

The experiments described here were designed: (1) to determine the time at which v^+ hormone appears in the optic anlagen of wild type and brown flies; (2) to measure the amount of hormone present in the optic discs at various stages of development after its appearance there; and (3) to test directly, by means of transplantation, the production of v^+ hormone by wild type optic anlagen.

MATERIALS AND METHODS

The techniques used for collection of pupae, transplantation, and injection of extracts are essentially those described by Ephrussi and Beadle (1936). Ages of larvae were measured from egg laying (eggs collected over a four-hour period), those of pupae from puparium formation, and those of adults from emergence. All cultures were kept at 25° C. Wild type flies used were F_1 offspring of a cross of the wild type stocks Canton-S by Oregon-R.

Extracts of optic discs were prepared in the following manner: After dissection from the animals, optic anlagen were transferred to 0.01 cc. of Ringer's solution contained in a small glass tube. The latter was heated (after the addition of each 5 pairs of discs) by partially immersing it in boiling water in order to destroy hormone-inactivating enzymes (Thimann and Beadle, 1937). The extracted discs were then separated from the fluid by centrifuging the fluid through a sintered glass filter fused into the bore of a small glass tube, the lower closed portion of which received the extract. The elongate receiving tube could then be separated from the filter, and, when desired, the contained extract could be hermetically sealed, sterilized, and stored. In most cases, however, extracts were injected at once. In all cases 0.01 cc. of Ringer's solution was used, and in most cases 25 pairs of optic discs extracted.

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[1] Part of a thesis submitted to the School of Biological Sciences of Stanford University in partial fulfillment of the requirements for the degree of Master of Arts.

Extracts were tested for quantity of hormone by injecting 1.4 mm.^{3} (the maximum volume practicable) into mature larvae of the standard test animal, vermilion brown, and the eye color of the emerged flies compared with the series of genetic standards developed and described by Tatum and Beadle (1938).[2] The color modification of the eyes of injected test animals as measured by the scale of Tatum and Beadle (1938) varies logarithmically with the amount of hormone injected. Hence, color values can be converted directly into arbitrary units of hormone. The amounts of hormone contributed per donor fly were then calculated and are listed in Tables I, II, and III as "units per donor fly."

Optic disc extracts were made from: (1) wild type larvae approximately 120 hours old; (2) brown and wild type pupae of various ages; and (3) brown and wild type adults of various ages. In the transplant experiments, wild type optic discs from larvae close to puparium formation were transplanted into vermilion brown larvae of the same age. Transplants were recovered from the hosts on emergence, extracts prepared and tested as indicated above.

Experimental

Extracts of Larval, Pupal, and Adult Eyes

From tests of three extracts, one made from 40 pairs and two from 100 pairs of larval discs, no eye color modification showed in the 12 test flies recovered. Hence it can be concluded that the hormone is not present in the eyes in detectable amounts before puparium formation.

Table I summarizes the data obtained from extracts of pupal eyes at various stages of development. In both brown and wild type, the hormone first occurs in the anlagen between 35 and 40 hours after puparium formation. The maximum color modification for each extract (shown in Fig. 1) is plotted against the age of donors after puparium formation. It is seen that the two series are similar; the amount of hormone increases rapidly during the first 15 to 20 hours after its appearance, reaching, in the case of wild type, a maximum at 3.5, which is maintained until emergence. In the brown series this value of 3.5 was obtained twice, i.e. for 80-hour pupae and for emerging flies; other maximal values obtained during pupal development range from 2.8 to 3.0. However, because the differences between the high values of brown and of wild type are no greater than the variations in the wild

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[2] No standards were available for values between 2.0 and 3.5; thus classifications from about 2.4 to 3.0 may not be accurate.

TABLE I

Production of v⁺ hormone by the eyes of wild type and brown pupae.

Donors		No. of Hosts		Color Values		Units per donor*
Age in hours	Number	Injected	Emerged	Range	Mean	
Wild type						
31	25	6	4	0.0	0.00	0.00
31	25	6	5	0.0	0.00	0.00
36	50	5	4	0.0	0.00	0.00
36	90	9	6	0.0	0.00	0.00
42	32	5	3	0.2–0.4	0.30	0.14
48	25	7	6	1.1–1.5	1.10	0.47
50	25	8	7	0.8–1.6	1.14	0.48
50	25	5	2	2.2–2.4	2.30	1.03
51	23	6	6	0.6–2.0	1.17	0.79
55	25	6	6	1.2–2.0	1.70	0.72
55	25	6	3	0.7–2.0	1.57	0.72‡
60	25	7	5	2.3–3.5	3.03	2.66
60	25	7	7	1.0–2.2	1.86	0.88
60	25	6	4	1.2–1.8	1.57	0.61‡
65	25	8	6	1.8–2.2	2.07	0.88
65	25	5	4	1.5–2.0	1.87	0.72‡
70	25	9	8	0.8–3.5	2.18	2.66
70	25	6	2	2.0–3.0	2.50	1.70†
70	25	6	4	2.0	2.00	0.72‡
75	25	9	8	1.2–3.5	1.98	2.66
75	25	7	1	2.8	2.80	1.44†
80	25	8	7	1.5–3.0	2.14	1.70
90	24	6	1	3.5	3.50	2.74†
91	25	7	7	3.0–3.5	3.36	2.66
95	25	9	3	2.0–3.5	3.00	2.66

TABLE I (*Continued*)

Donors		No. of Hosts		Color Values		Units per donor*
Age in hours	Number	Injected	Emerged	Range	Mean	
Brown						
35	25	7	6	0.0	0.00	0.00
40	25	6	5	0.0–0.2	0.10	0.16
45	25	5	2	0.5	0.50	0.19
50	25	6	4	1.5–2.0	1.78	0.72‡
51	25	7	6	0.8–2.1	1.33	0.84
55	25	5	5	2.0–2.8	2.36	1.44
55	25	9	8	1.5–3.0	1.98	1.70
61	25	6	5	1.1–2.4	1.98	1.03
61	25	5	1	1.7	1.70	0.51†
65	25	7	6	1.5–2.8	2.13	1.44
65	25	10	8	0.8–2.0	1.83	0.72§
70	25	6	6	2.1–2.8	2.52	1.44
70	25	6	3	0.8–2.0	1.53	0.72‡
70	25	4	1	1.5	1.50	0.47†
70	25	6	1	1.2	1.20	0.37†
75	25	8	5	2.1–2.8	2.44	1.44
75	25	5	3	2.3–2.5	2.40	1.13
75	25	7	1	2.0	2.00	0.72†
80	25	6	4	2.5	2.50	1.13‡
80	25	6	4	0.8–3.5	2.83	2.66
85	25	6	2	2.2–3.0	2.60	1.70‡
90	25	7	5	1.0–2.0	1.60	0.72‖
95	25	7	4	2.5–3.2	2.85	2.03
95	25	6	4	1.5–3.0	2.50	1.70

* Values calculated from the maximal color modifications.

† Not reliable statistically.

‡ Some hormone may have been destroyed by overheating when sealing off the glass tube for storage of the extract.

§ Extract may have been too dilute.

‖ Some hormone may have been lost during dissection.

type values themselves, it is probable that production of hormone is essentially the same in both cases; and therefore the same curve is drawn for both brown and wild type (Fig. 1).

In both series a few values seem to be either too high or too low as compared to the majority. Various reasons may be given as possible explanations for these aberrant color values. First, injury to the discs and prolonged exposure in Ringer's solution during dissection may account for some loss of hormone; second, although hormone extracts may be kept indefinitely when properly sealed off in glass tubes (Tatum, unpublished), care must be taken to avoid overheating and evaporation, which may have occurred in some instances. A third source of error may be simply a statistical one. In some experiments the larval mortality was unusually high due possibly to bacterial infection. These cases, when only one fly emerged, may not be representative of a normal amount of injected hormone, since some variability in the amount in-

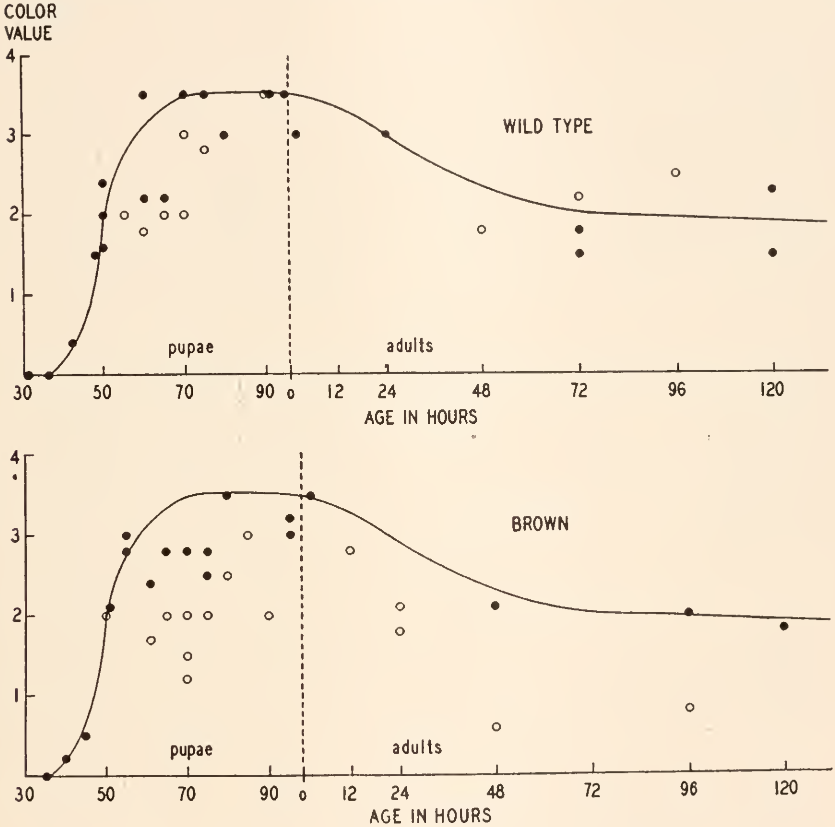

FIG. 1. Measurement of v^+ hormone in wild type and in brown eyes at various stages of development. (Experimental errors may reduce but not increase the apparent amount of hormone. Therefore the maximal values are considered more significant than the average, and the curves are plotted accordingly.)

jected is inherent in the technique. Values believed to be unreliable for one or more of the above reasons are indicated in open circles in Fig. 1.

TABLE II

Amount of v^+ hormone present in eyes of wild type and brown flies.

Donors		No. of Hosts		Color Values		Units per donor*
Age in hours	Number	Injected	Emerged	Range	Mean	
Wild Type						
1–4	25	?	6	2.5–3.0	2.83	1.70
24	25	6	2	2.9–3.0	2.95	1.70
48	25	8	7	0.8–1.8	1.53	0.61§
72	25	7	5	1.0–1.5	1.34	0.47
72	25	9	6	0.5–1.8	1.33	0.61
72	25	4	2	1.8–2.2	2.00	0.88‡
96	25	4	2	2.0–2.5	2.25	1.13‖
120	25	8	5	1.5–2.3	1.82	0.94
120	25	8	5	0.8–1.5	1.02	0.47
144	25	5	3	1.8–2.0	1.87	0.72
Brown						
1–4	25	5	2	3.4–3.5	3.45	2.66
12	25	8	4	2.0–2.8	2.35	1.44‡
24	25	5	1	2.1	2.10	0.85†
24	25	7	3	1.0–1.8	1.43	0.61‡
48	25	6	2	2.0–2.1	2.05	0.85
48	25	9	2	0.4–0.6	0.50	0.21‡
96	25	5	2	2.0	2.00	0.72
96	25	6	3	0.4–0.8	0.57	0.25‡
120	25	6	4	1.5–1.8	1.70	0.61
120	25	6	5	1.7–1.8	1.76	0.61
144	25	6	2	1.0–1.5	1.25	0.47
144	25	6	3	0.0–0.5	0.23	0.19‡
168	25	6	2	0.7–1.3	1.00	0.40

* Values calculated from the maximal color modifications.

† Not reliable statistically.

‡ Some hormone may have been destroyed by overheating when sealing off the glass tube for storage of the extract.

§ Some hormone may have been lost during dissection.

‖ Extract may have been too concentrated.

The results for extracts of adult eyes (Table II and Fig. 1) indicate a significant decrease in the amount of hormone only during the first few days after emergence. The value obtained from wild type flies shortly after emergence seems to be low, but because the data were obtained from only a single extract, the value may not be significant.

It should be noted that data from single extracts are not quantitatively accurate, but the trend of the whole series is believed to be significant.

Extracts from Wild Type Eyes Grown in Vermilion Brown Hosts

Whether or not eye tissues themselves produce v^+ hormone was tested by transplanting optic discs from wild type larvae close to puparium formation into vermilion brown hosts of the same age, which are incapable of producing this hormone. Since it is known that the implants contain no hormone at the time of the operation (see above), its subsequent appearance would indicate production by the implant organ itself. After emergence of the hosts the implant eyes were recovered and extracted. The results of injection of these extracts show that the transplanted eyes had produced v^+ hormone (Table III).

TABLE III

Production of v^+ hormone by transplanted wild type eyes.

No. Eyes Recovered	No. of Hosts		Color Values		Units per* donor
	Injected	Emerged	Range	Mean	
50	7	5	0.8–2.2	1.55	0.88
50	7	1	2.0	2.00	0.72

* Values calculated from the maximal color modifications.

DISCUSSION

The results described above are in reasonable agreement with those of Tatum and Beadle (1938) in which dried whole pupae were extracted. For example, they recovered about 8 units of hormone per pupa, while the maximum amount obtained here was 3.36 units per pair of eyes. Other work has shown that the lymph (Beadle, Clancy, and Ephrussi, 1937), fat bodies and Malpighian tubes (Beadle, 1937) also contain the hormone. These sources may account for most of the difference in units noted.

Ephrussi (1938) discusses the general question of storage of insect hormones in organs and their utilization during specific developmental stages, called "sensitive" or "effective periods." The evidence from these experiments bearing on this question indicates that v^+ hormone is not stored as such in eye tissues for any appreciable time before pigment appears, since detectable amounts of hormone appear at 40 hours and pigmentation at 47 hours after puparium formation, respectively.

Since the development of pigment in the eye is dependent upon the presence of the hormone, it is of interest to compare the time relations of hormone production and pigment formation. From the time of its appearance to about 55 to 60 hours after puparium formation pigmentation appears to be similar in wild type and brown. A light yellow color appears between 46 and 48 hours; this darkens to tan and finally to brown at about 60 hours. Referring to Fig. 1, it is evident that the amount of hormone increases greatly during this period, and at 60 hours, reaches a maximum which is maintained until emergence; possibly an equilibrium is reached with the eye using the hormone as rapidly as it is produced.

The experiments in which wild type optic discs were grown in vermilion brown hosts show that they produce only about one-third as much hormone as can be extracted from discs in their normal environment. This result is not inconsistent with the conclusion of Ephrussi and Chevais (1938) that a wild type eye grown in a vermilion host cannot itself produce enough hormone to attain full wild type pigmentation. On the other hand, if a wild type disc cannot produce enough hormone to complete its pigmentation (e.g. wild type in vermilion), the question arises as to why any hormone can be extracted from it. No explanation for this can be offered, but it is suggested that the utilization of hormone for pigment formation may depend upon a threshold concentration.

Acknowledgment

The author is grateful to Professor G. W. Beadle, Dr. E. L. Tatum, and Mr. C. W. Clancy for their advice and assistance both in carrying out the work and in the preparation of the manuscript.

Summary

Ringer's extracts of eye anlagen of wild type and brown stocks show that v^+ hormone appears in the optic discs approximately 40 hours after puparium formation (6 to 8 hours before onset of visible pigmentation).

The trend of the measurements of the amount of hormone in pupal discs during the course of development indicates that after its appearance the amount increases rapidly to a maximum which is probably maintained until emergence.

Adult eyes show a decrease in amount of hormone with increasing age.

Extracts of transplanted discs show that wild type eyes actually produce v^+ hormone, but in amounts insufficient for the development of full pigmentation.

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COMPARATIVE RICHNESS OF ZOÖPLANKTON IN COASTAL AND OFFSHORE AREAS OF THE ATLANTIC

GEORGE L. CLARKE

(From the Biological Laboratories, Harvard University, and the Woods Hole Oceanographic Institution [1]*)*

Between our eastern coast and Bermuda lie three zones of water which are of great biological interest because of their extreme contrast: the coastal water, overlying the continental shelf; the "slope" water, which is the area of mixing between the continental slope and the Gulf Stream; and the Sargasso water, of which the Gulf Stream is the western and northern edge. An opportunity to make a comparative investigation of the zoöplankton of the coastal zone and of the zones farther offshore presented itself during the cruises of the research vessel "Atlantis" between Montauk Point, Long Island, N. Y. and Bermuda, which were begun in 1937.

Since the primary object of these cruises was a study of the physical structure of the Gulf Stream and since the ship was working in other localities during the intervals between these cruises, the plankton hauls were necessarily "catch-as-catch-can." Although an extensive network of stations throughout the region which were revisited at frequent intervals would obviously have been desirable, the data available from a single line of stations repeated ten times between October, 1937 and June, 1939 are instructive and raise certain important questions, especially when compared with earlier observations in this area.

Our first objective in the present investigation of the plankton was a comparison of the richness of the coastal and offshore zones. For this purpose four standard stations were located at approximately equal intervals across the coastal water and four more stations were occupied in the slope water beyond (Fig. 1). A ninth station located well within the limits of the Sargasso Sea was established as typifying oceanic water far removed from any influence of the coast. Since the abundance of the plankton was expected to vary within each zone during the course of the season, and since these variations probably would not be the same in different zones, a knowledge of the seasonal cycles of the plankton formed a second objective and one upon which the first necessarily depended. Furthermore, changes in the general nature and richness of the plankton might occur from year to year. It was therefore proposed to examine our data for annual differences in those months for which

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[1] Contribution No. 241.

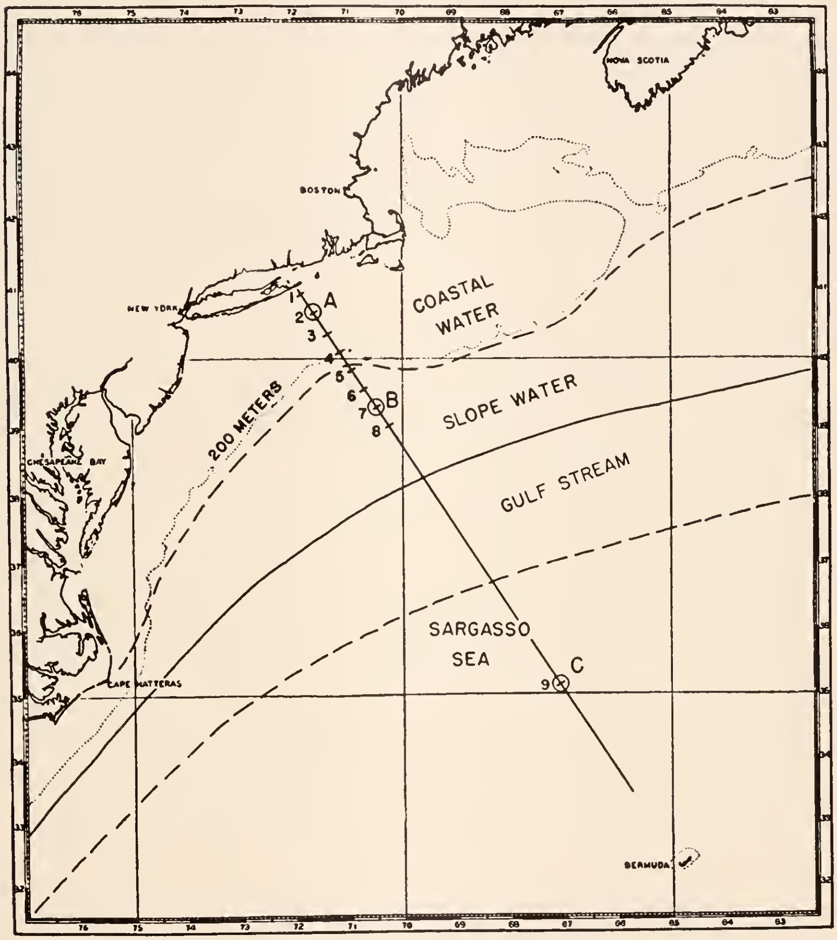
NOVA SCOTIA
BOSTON
NEW YORK
CHESAPEAKE BAY
CAPE HATTERAS
BERMUDA
COASTAL WATER
SLOPE WATER
GULF STREAM
SARGASSO SEA
200 METERS
A
B
C
1
2
3
4
5
6
7
8
9
76
75
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FIG. 1. *The location of stations in three areas of the Atlantic.* Stations 2, 7 and 9, at which certain extra observations were made, have been given the additional designation of *A*, *B*, and *C* respectively. The sharply delineated boundary of the Gulf Stream toward the coast is represented by a solid line; the more indefinite boundary toward Bermuda is indicated by a broken line.

we have observations in two years and also to compare our catches with those made by the U. S. Bureau of Fisheries in the coastal zone during 1929–32, which have been analyzed by Bigelow and Sears (1939).

HYDROGRAPHY

The coastal area is distinguished from the areas farther offshore first of all by the great and important difference in the depth of the water. At Station 1 the water averaged 44 meters deep, at Station 2(*A*), it averaged 69 meters, and at Station 3 it averaged 85 meters. Station 4 was located just at the brink of the continental slope and the slightly different positions reached on different cruises resulted in depths which varied from 159 meters to 375 meters. The depth in the slope water area was very much greater. At Station 5, located over the steep section of the continental slope, the depth varied from 411 meters to 1,134 meters and at the other stations in this area the water was from 2,000 to 3,000 meters deep. The depth at Station 9(*C*) in the Sargasso Sea averaged 5,100 meters.

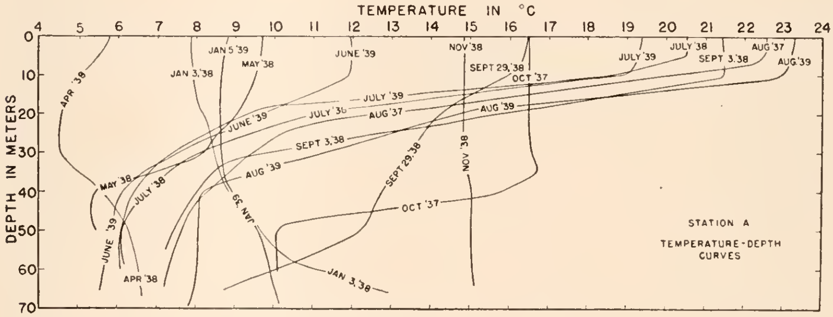

FIG. 2. *Coastal Water*—Seasonal changes in the distribution of temperature with depth at Station 2(*A*).

The currents in the areas under consideration are generally parallel to the coast in their broader aspect. In the coastal zone a slow and intermittent movement of the water down the coast in a southwesterly direction has been detected supplying Stations 1 to 4 with cooler and less saline water than exists offshore. In contrast, the western part of the Sargasso water, i.e., the Gulf Stream, is flowing rapidly in the opposite direction, and at Station 9(*C*) the highest temperatures and salinities were encountered. The slope water is an area of mixing in which intermediate conditions are to be expected. For a complete analysis of the hydrographic situation reference should be made to Iselin (1940). A synopsis of the conditions from the biological viewpoint follows.

At Station 2(*A*), which we may take as typical of the coastal region, the water is found to be completely mixed early in the winter with the result that the temperature is uniform from surface to bottom (Fig. 2).

Cooling continues during the winter until a minimum of about 4° C. is reached in February (Bigelow, 1933). With the arrival of the spring freshets in March and April the salinity of the surface water near the coast is reduced and consequently further vertical mixing with the deeper more saline strata tends to be resisted. At the same time the heat received from the sun, which is increasing at this season, progressively warms the water layers nearest the surface in which the major portion of the radiation is absorbed (Clarke, 1939*a*). Our first spring observation, in April, exhibits the beginning of the vernal warming of the uppermost layers. As this process continues on into the summer, vertical mixing is more and more retarded because of the increasing stability resulting from the greater differences between the surface and the bottom temperatures. Thus, in the late summer of our years, when the temperature at the surface reached the maximum value of about 23° C., water of only about 8° C. existed at the bottom and a sharp thermocline was to be found at mid-depths. During the autumn months cooling at the surface in combination with the stronger winds of that season results in progressively deeper stirring and the consequent further warming of the bottom layers until the winter condition of uniform temperatures is attained. The temperatures observed at Station 2(*A*) indicate that during the period of the present investigation the coastal water was neither abnormally warm nor abnormally cold during any season as judged by the records of previous years (Bigelow, 1933). The salinity at this station varied between $31.2^0/_{00}$ and $33.2^0/_{00}$ at the surface and between $32.7^0/_{00}$ and $33.9^0/_{00}$ at the bottom during the year. It is doubtful if these relatively slight differences in salt content are of any biological importance. The transparency of the water as measured by the percentage absorption of light per meter ranged from 10 per cent to 14 per cent (Clarke, 1939).

In the Sargasso Sea, as exemplified by Station 9(*C*), the depth to which the seasonal changes in temperature extend is much greater, but the range of temperature variation is much smaller than in the coastal area (Fig. 4). In the Sargasso water a "primary" thermocline extending from about 500 meters to 1100 meters is in existence throughout the year. The upper boundary of this "thermocline layer" represents the lower limit of the stirring action of the wind, and during the winter and early spring months the temperature is found to increase but little from this depth to the surface. Below 800 meters a temperature of less than 15° C. is to be found at all seasons (Iselin, 1936). In our present investigation a minimum temperature for the surface of about 19° C. was observed in April. The vernal warming which follows this winter condition not only raises progressively the temperature of the surface, but also produces during the ensuing months a *"secondary"* thermocline which is found first at 40 meters but later in the summer at nearly 100 meters. This secondary thermocline disappears again during the late autumn months as a consequence of the deeper stirring of that season. During the present investigation the surface layer attained a maximum temperature of nearly 27° at the end of August. Thus, the seasonal range at the surface amounted to only 8 degrees in contrast to the 15-degree change observed at Station 2(*A*). The salinity in the Sargasso area is remarkably constant: in the present investigation all observations down to 300 meters were confined between 36.1 and $36.7^0/_{00}$. The absorption of light by the water, which has been found

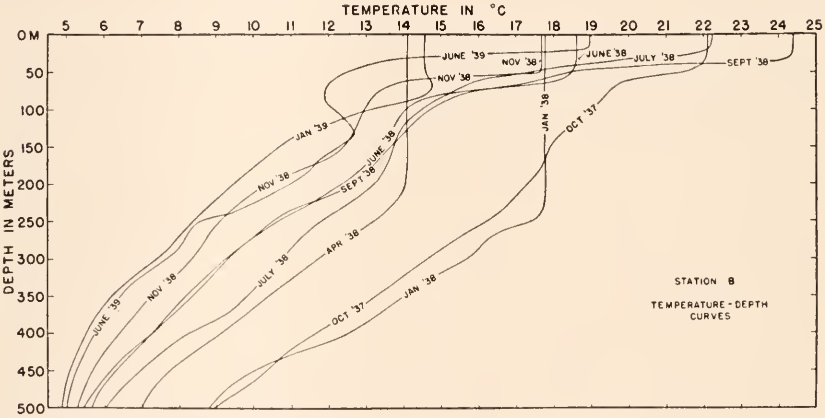

FIG. 3. *Slope Water*—Seasonal changes in the distribution of temperature with depth at Station 7(*B*).

to take place at an extremely low rate in this part of the ocean, varied between 4.2 per cent and 6.6 per cent per meter (Clarke, 1939*a*).

At Station 7(*B*), in the middle of the slope water zone, the hydrographic conditions were generally intermediate between those obtaining in the coastal and in the Sargasso zones. They are subject, however, to very large fluctuations, especially in the upper 200 meters, because nearly pure coastal water or nearly pure Sargasso water may be present at the station in question, and on our various visits all degrees of mixing were encountered. The seasonal temperature cycle is of the same nature here as in the Sargasso zone, but the main thermocline layer is shoaler, extending in the present case from about 250 meters to 600 meters and temperatures are generally lower (Fig. 3). Thus, at Station 7(*B*) the seasonal change of 10 degrees which was observed for the surface water ranged from 14° C. to 24° C. The salinity and the transparency of the slope water similarly veer toward values characteristic of the coastal water or of the Sargasso water, according to the proportions in which those two types are present as has been discussed in detail elsewhere (Clarke, 1939*a*).

Collection and Measurement of Plankton [2]

Our present plankton studies are based primarily on a pair of "oblique" hauls (one shallow and one deep) made with an open scrim net (10 strands/cm.) at each of the nine stations shown in Fig. 1. For the shallow haul the net was lowered to 25 meters in most cases (but to 50 meters at Stations 7(*B*) and 9(*C*)) and hauled in at the rate of 5 meters every 5 minutes (5 meters every 2 minutes from 50-meter level), while the ship was steaming at 1½ to 2 knots. For the deep haul, the net was lowered to the bottom at Stations 1–4, to about 275 meters at Stations 5–8, and to about 800 meters at Station 9, and then, while steaming, hauled up to 25 meters (or to 50 meters) at such a rate that

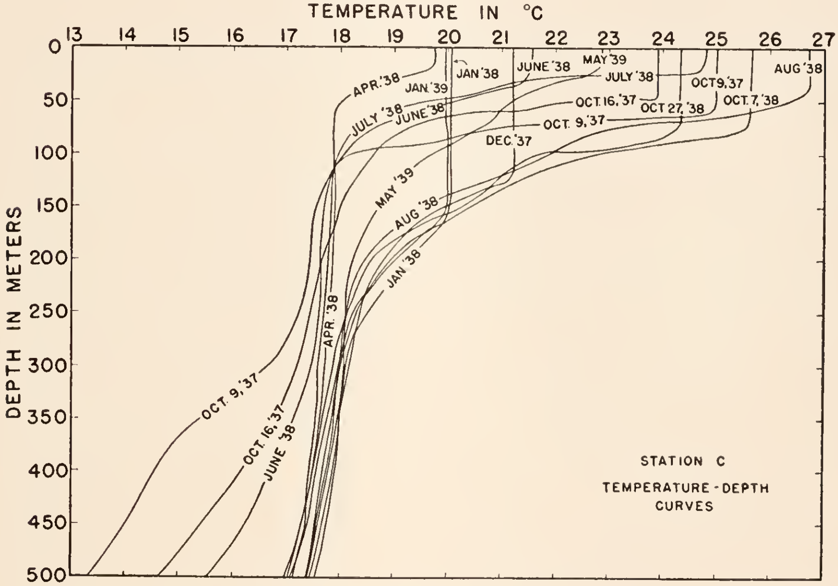

Fig. 4. *Sargasso Sea*—Seasonal changes in the distribution of temperature with depth at Station 9(*C*).

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[2] Carried out by Mr. Dean F. Bumpus, Biological Technician for the Woods Hole Oceanographic Institution.

the total towing period consumed about 30 minutes. In this way a picture was obtained of the plankton in the superficial stratum above the summer thermocline and of the plankton in the underlying strata from which some biological relationship with the surface might be expected.

At Stations *A*, *B*, and *C*, located within the three areas under consideration, additional observations were undertaken to supply further detail to the biological picture. Shallow and deep hauls with a stramin net (either 1½ or 2 meters in diameter) were made at these stations for the collection of the larger and less abundant elements of the population which might not be sampled adequately by the smaller nets. In these hauls the same depths and general procedure were used as for the work with the scrim nets. When weather permitted, both the scrim and the stramin nets were rigged for closing (Leavitt, 1935, 1938) as a check on the deductions from the open net hauls as to the exact nature of the vertical distribution of the zoöplankton. Those elements of the zoöplankton which were too small to be retained by the relatively coarse scrim net were investigated at these stations by means of shallow and deep oblique hauls with a No. 10 (43 strands/cm.) silk net 30.5 cm. in diameter. In addition, phytoplankton material was obtained at the three key stations, and in some cases at the intervening stations as well, from water samples and from hauls with No. 20 silk nets, and was turned over to Dr. Lois Lillick and to Dr. James B. Lackey for study.

For the whole of the cruise of May 29–June 5, 1938, for the major part of the cruise of July 9–17, 1938, and in certain other cases, hauls were made with plankton samplers provided with No. 10 silk nets or with No. 2 (21 strands/cm.) silk nets. The plankton sampler is a newly developed instrument consisting of a tube 12.7 cm. (5 inches) in diameter to the end of which a conical net is attached. The tube is provided with a shutter and contains a volume meter which records the amount of water which has actually passed through the net (Clarke and Bumpus, 1939). Tests reported by Winsor and Clarke (1940) indicate that a net with a diameter as small as 12.7 cm. is as reliable as a net 75 cm. in diameter. The operation of the samplers, in their earlier design, was interfered with by the unusual abundance of salpae which were encountered at a large number of the stations (see below), and it was found that the towing periods employed in the barren offshore waters were not long enough to yield a catch of satisfactory size for detailed analysis. Nevertheless, the work with the plankton samplers provides us with a certain number of cases from which the absolute density of the plankton under various circumstances can be deduced with precision. The plankton sampler hauls therefore comprise a valuable adjunct to the hauls with nets of the more usual type.

The abundance of the zoöplankton taken in the hauls has been determined in all cases by means of volume measurement and, in the case of certain species, by enumeration. The volume was measured by displacement, since Bigelow and Sears (1939) have recently called attention to the superiority of this procedure over the settlement method. The whole catch was placed in a graduated cylinder and the total volume noted. The catch was then poured through netting of the same (or of smaller) mesh than that of the net used in making the catch and allowed to stand until dripping had practically stopped. The volume of water thus separated was then measured and subtracted from the total volume to obtain the volume of the plankton alone. In those cases in which sagittae, salpae, or coelenterates formed an important fraction of the total catch, these elements were picked out and measured separately. The remaining plankton, composed chiefly of copepods and euphausids but containing also some amphipods, limacina, etc., has been designated as the "crustacean" fraction of the haul.

The species in the present study which were enumerated were the important copepods, *Calanus finmarchicus* and *Centropages typicus*. Separate count was kept of the males and females and, in the case of *Calanus*, of the developmental stages as well. The method employed for sub-sampling and for counting[3] was that described by Winsor and Clarke (1940).

Variability in Volume and in Type of Plankton

The volumes of all the hauls made with the scrim net in the present investigation are presented in Tables I and II, which give respectively the values for the "total plankton" and for the "crustacean plankton." It will be observed that the amounts of plankton caught are extremely variable, ranging from a maximum of 3,060 cc. to a minimum of 1 cc. in the case of the total plankton and from 225 cc. to 1 cc. for the crustacean plankton. Very considerable changes in volume were often encountered at neighboring stations, as was also observed by Bigelow and Sears (1939). This situation immediately raises the question of the reliability of single hauls. Fortunately there is available a statistical study of the variation to be expected from just such material as is dealt with here in the report of Winsor and Clarke (1940). These investigators found that in the case of oblique hauls with a 75 cm. scrim net, the standard deviation of the haul-to-haul variation was about 20 per cent of the mean. It is therefore obvious that the major portion, at

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[3] Several small hand "counting machines" mounted on a board were found to be extremely useful for keeping count of the various categories observed through the microscope.

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TABLE I

Total plankton. Volume in cc. of plankton of all types taken with 75 cm. scrim net. Volume reduced to 30-minute haul. Hauls in italics taken between sunset and sunrise. *S* = shallow. *D* = deep (see text).

	Station	Depth	1938 Jan. 2–7	1939 Jan. 5–10	1938 Apr. 6–13	1939 May 28–June 3	1938 July 9–17	1938 Aug. 28–Sept. 3	1938 Sept. 29–Oct. 8	1937 Oct. 2–9	1937 Oct. 17–21	1938 Oct. 27–Nov. 3	Average
Coastal Water	1	*S*	*26*	—	—	62	85	8	—	*45*	—	*178*	
		D	*36*	—	—	64	*100*	15	—	*150*	—	*100*	
	2(*A*)	*S*	*20*	—	*24*	*84*	107	220	35	*81*	*20*	400	
		D	*26*	—	4	*55*	214	970	—	*115*	*27*	114	
	3	*S*	31	*4*	40	*162*	—	*83*	*660*	265	—	*190*	
		D	20	*7*	—	*222*	3060	*202*	*220*	262	—	*129*	
	4	*S*	5	—	170	22	—	*720*	*54*	225	—	*115*	
		D	5	—	—	94	—	*590*	*82*	308	—	*53*	
	Average	Shallow	21	4	78	83	96	258	249	154	20	221	118
	Average	Deep	22	7	4	109	1125	444	151	205	27	99	220
	Combined	(Weighted)	22	7	23	103	868	398	176	192	25	129	194
Slope Water	5	*S*	*2*	*22*	9	17	—	—	—	*435*	—	—	
		D	10	*40*	*40*	22	—	—	—	*200*	—	—	
	6	*S*	*31*	9	*17*	60	—	12	30	*82*	—	40	
		D	*13*	11	*43*	254	—	20	20	*111*	—	26	
	7(*B*)	*S*	7	16	*32*	*515*	—	5	*40*	*130*	75	*145*	
		D	*6*	38	*8*	*54*	—	18	*55*	*155*	22	*32*	
	8	*S*	*11*	*375*	*5*	31	—	*85*	9	80	—	60	
		D	*11*	*113*	*10*	44	—	*100*	—	48	—	53	
	Average	Shallow	13	106	16	156	—	34	26	182	75	82	77
	Average	Deep	10	51	25	99	—	41	38	129	22	37	50
	Combined	(Weighted)	10	56	24	104	—	40	37	134	27	41	52
Sargasso Sea	9(*C*)	50–0 m.	*55*	9	*33*	*15*	—	*9*	*25*	*45*	*10*	*27*	29
		100–50	—	14	*53*	*43*	—	*38*	*27*	—	*5*	*12*	27
		500–100	*5*	*18*	*13*	—	—	*6*	*9*	*7*	*4*	*5*	8
		900–500	*4*	—	*5*	—	—	*1*	*9*	—	*6*	*5*	5
	Combined	(Weighted)	7	17	13	29	—	6	11	11	5	7	12

TABLE II

Crustacean plankton. Volume in cc. of plankton after removal of major portion of sagittae, salpae and coelenterates taken with 75 cm. scrim net. Volume reduced to 30-minute haul. Hauls in italics taken between sunset and sunrise. *S* = shallow, *D* = deep (see text).

	Station	Depth	1938 Jan. 2–7	1939 Jan. 5–10	1938 Apr. 6–13	1939 May 28–June 3	1938 July 9–17	1938 Aug. 28–Sept. 3	1938 Sept. 29–Oct. 8	1937 Oct. 2–9	1937 Oct. 17–21	1938 Oct. 27–Nov. 3	Average
Coastal Water	1	*S*	*26*	—	—	62	37	8	—	*38*	*20*	*56*	
		D	*20*	—	—	64	*71*	—	—	*126*	*27*	*50*	
	2(*A*)	*S*	*20**	—	*15*	*84*	91*	52	14	*46**	—	50	
		D	*26**	—	4	*55*	214	156	—	*62**	—	48	
	3	*S*	24	*4*	1	*162*	—	*45*	*0*	85	—	*74*	
		D	20	*7*	—	*126*	68	*47*	*22*	86	—	*52*	
	4	*S*	—	—	84	11	—	*135*	*10*	25	—	*70*	
		D	4	—	—	73	—	*225*	*22*	103	—	*16*	
	Average	Shallow	23	4	33	80	64	60	12	48	20	62	41
	Average	Deep	18	7	4	79	118	143	22	94	27	42	50
	Combined	(Weighted)	19	6	11	79	104	122	19	82	25	47	50
Slope Water	5	*S*	*2*	*18*	8	9	—	—	—	*21*	—	—	
		D	10	*40*	*40*	11	—	—	—	*12*	—	—	
	6	*S*	*16*	9	*17*	15	—	1	12	*45*	—	4	
		D	*8*	11	*39*	18	—	16	*7*	*39*	—	11	
	7(*B*)	*S*	5	13	*32*	*72**	—	3	*14**	*45*	4	*68**	
		D	*6*	25	*8*	*43**	—	14*	10*	*23*	12	*16**	
	8	*S*	*7*	*28*	*3*	1	—	*76*	9	15	—	14	
		D	*10*	*56*	*10*	9	—	—	—	20	—	15	
	Average	Shallow	8	17	15	22	—	27	12	32	4	29	18
	Average	Deep	9	33	24	22	—	15	9	24	12	14	18
	Combined	(Weighted)	9	32	23	22	—	16	9	25	11	15	18
Sargasso Sea	9(*C*)	50–0 m.	*25**	5	*19**	*10*	—	*6*	*16*	*44*	*5*	*21*	17
		100–50	—	10	*42**	*16*	—	*24*	*19*	—	*4*	*6*	17
		500–100	*3*	*13*	*5*	—	—	*4*	*5*	*6*	*3*	*2*	5
		900–500	*4*	—	*4*	—	—	*1*	—	—	*4*	*2*	3
	Combined	(Weighted)	5	12	7	13	—	4	8	10	4	4	7

* Occasions on which the small copepods in the corresponding No. 10 silk net haul amounted to > 5 cc.

least, of the fluctuation in volume observed in the present case is real and not due to errors of observation.

A further question, and one more difficult to answer, is the extent of the area over which each haul may be taken as representative. Previous plankton studies have shown that the dispersal pattern of the population in each situation must be investigated individually. For this purpose a closer network of stations must be arranged than was possible in the present case and strictly quantitative hauls employed, as, for example, through the use of registering plankton samplers (Clarke and Bumpus, 1939) or of a continuous plankton recorder (Hardy, 1936).

TABLE III

Comparison of three sets of 30-minute hauls with 75 cm. scrim net at Station 2(*A*) as a sample of variability.

	Oct. 2, 1937 Closing net (Day Haul)	Oct. 2, 1937 Open net (Day Haul)	Oct. 3, 1937 Open net (Night Haul)
Total volume (cc.)			
Shallow	33	140	45
Deep	46	127	150
Total	79	267	195
Crustacean volume (cc.)			
Shallow	33	14	38
Deep	46	58	126
Total	79	72	164
Calanus stage IV			
Shallow	250	234	400
Deep	1,940	3,000	6,500
Calanus stage V			
Shallow	500	117	900
Deep	2,500	7,200	16,200
Centropages ♂			
Shallow	8,500	5,050	5,800
Deep	21,600	24,600	15,500
Centropages ♀			
Shallow	22,200	9,800	14,800
Deep	51,000	41,100	28,500

In the present investigation two bits of evidence exist on the variability of hauls closely spaced in time and in position. At Station 2(*A*) on October 2, 1937, a pair of hauls with the closing scrim net was followed immediately with a pair of hauls of the same type with the open net and in addition the open net hauls were repeated at this station on the following day (Table III). In each of the categories compared in this table, the largest volumes are found to be three or four times greater than the smallest volumes. In those cases in which numbers of individuals are compared, the differences are greater. Evidently the variations of the separate groups considered tend to cancel out when volumes of mixed species are compared.

A more extensive study of the variation encountered within a small area was made on May 17–18, 1938, when hauls were made with the scrim net and with the plankton samplers (No. 2 silk) at Station 2(*A*) and at four stations located about 10–15 miles distant to the south, east, north, and west respectively of Station 2(*A*). The work with the plankton samplers was repeated at Station 2(*A*) on May 30, 1938. The variation in the volumes of these hauls and in the number of *Calanus* in copepodid stages IV and V may be observed in Table IV. Not only did the volumes fluctuate irregularly from station to station with differences as great as 10-fold in extreme cases, but also it is seen that the variations in the shallow stratum did not conform to those in the deeper layers. As in the previous case, the fluctuations in the actual numbers of *Calanus* are even greater than the changes in volume. The histogram plots of Figs. 5 and 6 reveal the very great extent to which the species composition of these hauls varies. The diagrams show, for example, that at some of the stations in this group *Calanus* was the dominant form in the catches of the scrim net, whereas at other stations small medusa were by far the most abundant organisms. Similarly, in the case of the hauls with the plankton samplers, the proportion in which each type of animal is represented varies tremendously—in one haul *Calanus* takes the lead, in other hauls *Evadne, Pseudocalanus,* or medusae greatly outnumber all the other forms.[4] In view of this far-reaching irregularity in distribution, it is essential that the number of stations occupied be as large as possible and that all hauls within each area be combined in suitable ways before any conclusions be attempted in regard to relative richness. In the following discussions, therefore, *average values* will be considered for the most part for each of the areas under consideration.

In all the foregoing data it is evident that the variability of " crustacean volume " is much less than that of the " total volume." We may conclude, therefore, that fluctuation in the abundance of salpae, sagittae, coelenterates and other non-crustacean elements are largely responsible for the great irregularities of Table I. The more uniform complexion of the hauls during the winter months is largely due to the fact that the

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[4] The dominant species were not expected to be the same as in the scrim net hauls since the netting in the plankton samplers was of smaller mesh.

TABLE IV

Comparison of scrim net hauls at Station 2(*A*) and at four surrounding stations 10–15 miles distant on May 17–18, 1938, and comparison of plankton sampler hauls for the same stations and for Station 2(*A*) on May 30, 1938.

	Scrim net (75 cm. diameter). Vol. or no. per 30 min. haul					Plankton sampler. Vol. or no. per m³. of sea water					
	Sta. 2(*A*) May 17, '38 (Day Haul)	Sta. 2(*A*)–*S* May 17, '38 (Night Haul)	Sta. 2(*A*)–*E* May 18, '38 (Night Haul)	Sta. 2(*A*)–*N* May 18, '38 (Day Haul)	Sta. 2(*A*)–*W* May 18, '38 (Day Haul)	Sta. 2(*A*) May 17, '38 (Day Haul)	Sta. 2(*A*)–*S* May 17, '38 (Night Haul)	Sta. 2(*A*)–*E* May 18, '38 (Night Haul)	Sta. 2(*A*)–*N* May 18, '38 (Day Haul)	Sta. 2(*A*)–*W* May 18, '38 (Day Haul)	Sta. 2(*A*) May 30, '38 (Night Haul)
Total volume (cc.)											
Shallow *	57	119	23	112	75	1.02	.94	.31	.71	.76	.34
Intermediate †						.48	.56	1.08	.29	.74	—
Deep ‡	21	120	112	13	26	1.90	.90	.96	1.02	1.15	—
Crustacean volume (cc.)											
Shallow	57	119	18	28	8						
Intermediate						—	—	—	—	—	—
Deep	21	105	25	11	26						
Calanus stage IV											
Shallow	31,500	29,400	7,550	12,700	1,900	785	161	202	475	310	87
Intermediate						84	218	111	100	167	32
Deep	4,950	26,700	7,500	1,940	2,420	69	27	218	101	96	66
Calanus stage V											
Shallow	16,300	22,200	4,580	7,750	2,050	108	75	24	35	38	73
Intermediate						42	175	174	44	53	45
Deep	6,400	32,800	10,500	4,750	5,500	57	113	115	82	32	10

* "Shallow" for both scrim net and plankton sampler indicates oblique haul from 25 meters (wire out) to surface.

† "Deep" for scrim net indicates oblique haul from bottom to 25 meters; for plankton sampler, bottom to 50 meters.

‡ "Intermediate" for plankton sampler indicates oblique haul from 50 meters to 25 meters.

plankton at that season consisted mainly of crustacea, particularly at stations toward the coast. Salpae, which were unusually abundant during the present investigation, not only in the slope water, where they are known to be common, but also in the coastal water, most frequently accounted for the difference between the crustacean volume and the total volume. Indeed, in every case but one, values greater than 200 cc. were attributable to the presence of salpae.

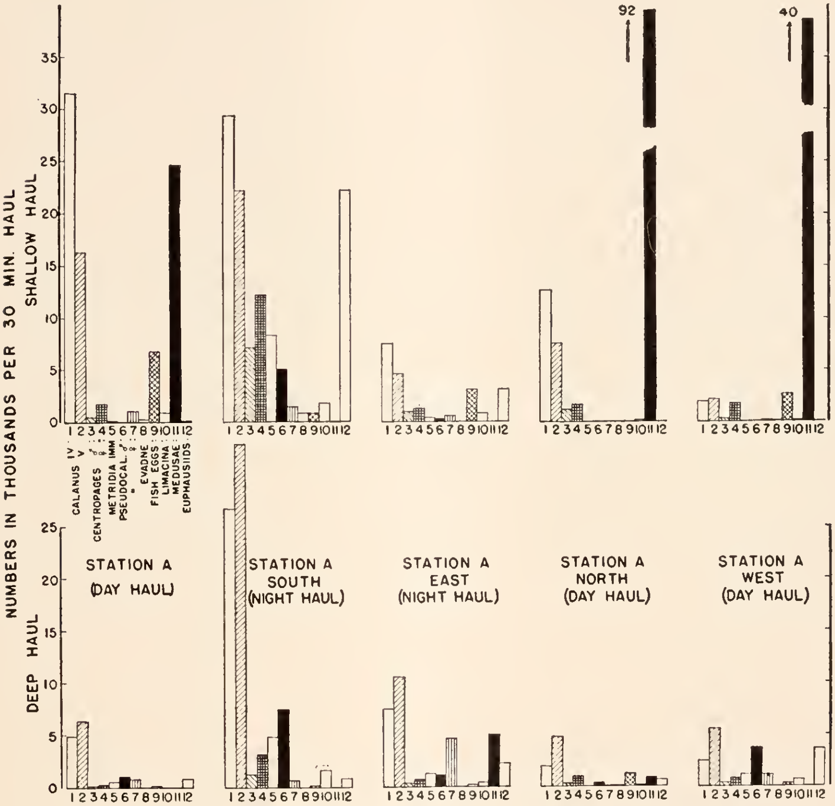

FIG. 5. Variation in composition of scrim net hauls at Station 2(*A*) and at four surrounding stations 10–15 miles distant on May 17–18, 1938.

The separation of the crustacea from the other types of animals provides us with an approximate division of the plankton into (1) the "more nutritive" forms which are known to be prominent in the diet of the important fish of the region and (2) the forms of little or no nutritional value. This method of subdivision conforms to that adopted by Bigelow and Sears (1939) with the exception that these authors included the sagittae with the crustacea as being of considerable food value. Since in the present investigation sagittae were relatively rare (occurring chiefly in the winter months) and in no case amounted to more than 50 cc., it appears legitimate in this case to regard our "crustacean volumes" as comparable to the category of "more nutritive forms" dealt with by Bigelow and Sears. The volumes appearing as "crustacean plankton" (Table II) may thus be accepted as a rough index of

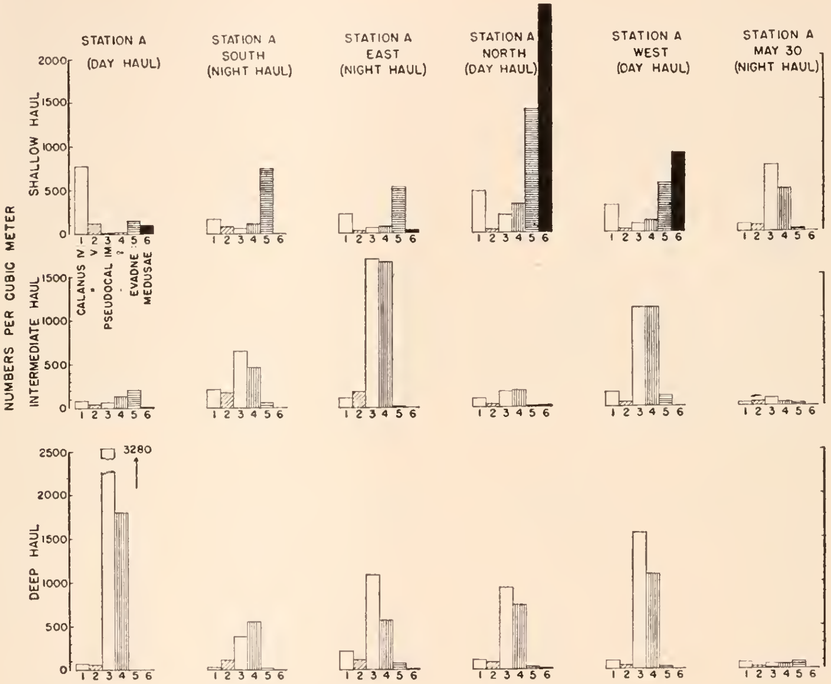

Fig. 6. Variation in composition of plankton sampler hauls at Station 2(*A*) and at four surrounding stations 10–15 miles distant on May 17–18, 1938 and at Station 2(*A*) on May 30, 1938.

the abundance of food for plankton-feeding fish within the bounds of the present investigation.

Hauls made with the large stramin nets and with smaller nets of No. 10 silk at Stations *A*, *B* and *C* were examined for the possible occurrence of important elements of the plankton which were either too large and active or too small to be caught adequately by the scrim nets. The volumes of plankton taken by the stramin nets were of the same order of magnitude as those taken by the scrim nets although the amount of water filtered by the former type of net was very much greater. Evidently the loss of the types of plankton which slip through the meshes of the stramin net is relatively greater than the additional number of active species which are taken by this net but missed by the scrim net. A certain portion of larger crustacea were found in the stramin net hauls but the bulk of the more voluminous catches consisted of salpae, as was the case with the scrim net hauls. At the other end of the size scale there exist members of the plankton community which are not retained even by the scrim net. Hauls made simultaneously with scrim and with fine silk nets of the same size (unpublished records) have shown that in situations where small copepods (such as *Pseudocalanus, Paracalanus,* or immature *Centropages*) are abundant, the catch of the silk net may be several times greater than that of the scrim net. Under such circumstances the volumes of scrim net hauls alone would greatly underrate the richness of the plankton. However, the hauls with the No. 10 silk nets indicate that the smaller animals were not generally abundant during the present investigation. Amounts of crustacean plankton greater than 5 cc. were taken in these nets on only three occasions at Station *A,* four occasions at Station *B,* and two occasions at Station *C,* as indicated in Table II. Volumes greater than 10 cc. were taken only at Station *A,* once in July and once in October. We may conclude, therefore, that for the investigation as a whole the scrim net sampled the major portion of the population and that the hauls with this net are a satisfactory index to the richness of the animal plankton.

DISTRIBUTION OF PLANKTON BY VOLUME

Vertical Distribution

The vertical distribution of the plankton in the three areas may be ascertained from Tables I and II, in which the volumes taken in the "shallow" and "deep" hauls at each station are compared. Since the time of visiting each station fell at various hours of the day or night, the influence of any diurnal vertical migrations on the picture presented must be considered (Clarke, 1933 and 1934). For this purpose the hauls made during the day between sunrise and sunset have been differentiated in the tables from those made during the night.

For the total plankton in the coastal area, the deep hauls were greater than the surface hauls in 62 per cent of the stations occupied during daylight hours and in 67 per cent of the night stations. In the slope water area the deep hauls were greater in 58 per cent of the day stations and in 54 per cent of the night stations. These figures indicate that the vertical distribution of the plankton as a whole is essentially the same by day or by night in these areas. When the crustacean plankton is considered separately, it is found that in the coastal area the deep hauls were greater than the surface hauls in 75 per cent of the day hauls and in 60 per cent of the night hauls. In the slope water area the deep hauls were greater in 100 per cent of the day hauls but in only 42 per cent of the hauls made during the night. These figures make it clear that a diurnal vertical movement of the crustacea was taking place in the slope water area and to a lesser extent in the coastal water. Unfortunately, all the hauls except two at Station 9(*C*) in the Sargasso Sea fell during the night period and consequently we are unable to state whether the distributional picture would have been different had a significant number of day hauls been made (cf. Waterman, Nunnemacher, Chace, and Clarke, 1939).

For a *general* consideration of vertical distribution in the coastal area the day and night hauls may be averaged since the differences between them are not great. The average volume of the total plankton of all the shallow hauls in this area was 118 cc. and of the deep hauls was 220 cc. If, however, the cruise of July 9–17, 1938 is omitted because of the presence in one haul of an exceptionally large number of salpae, these values become 120 cc. for the surface and 119 cc. for the deep hauls. The average volumes of the crustacean plankton in this area were 41 cc. for the shallow hauls and 50 cc. for the deep. In the slope water area day and night hauls may similarly be averaged for a general consideration since there are an equal number of each. In this area the total plankton averaged 77 cc. for the shallow hauls and 50 cc. for the deep hauls and the crustacean plankton averaged 18 cc. for the hauls at both levels. We may therefore conclude that for the year as a whole no important difference existed in the richness of the plankton in the upper and lower strata in these two areas. At the station in the Sargasso Sea, the volume of the crustacean plankton and of the total plankton was about the same in the uppermost 50 meters of water as it was in the stratum from 100 meters to 40 meters. But in every case except one (in which the shallower hauls were made during the day) the plankton was more abundant above 100 meters than in the deeper strata.

Seasonal Distribution

In order to obtain a value characteristic of the whole population in each area for each season and to eliminate any localized influence which diurnal migration might have on the distributional picture, we may combine the shallow and deep hauls for each of the cruises. It must be remembered, however, that at no station was it practicable to allow the net to fish down to the very bottom and at the deeper stations a considerable stratum remained unsampled. Although the possibility exists that a population of some importance might thus have been missed, we have no evidence that the plankton just over the bottom on the continental shelf is significantly more abundant than in the water nearer the surface. In the oceanic areas with which we are concerned here, Leavitt (1938) found that the macrozoöplankton was considerably less rich below 1,000 meters than above that depth. In combining the average hauls at the two levels, the deep haul has been weighted in proportion to the relative thickness of the stratum through which it was towed as compared with the shallow haul. The combined average is thus a characteristic index of the whole depth of water sampled.

Considering first the coastal area, we find that the plankton as a whole reached its lowest ebb during the winter in both 1938 and 1939 (Table I). By the end of May, the volume of the catches was greatly increased and in July and August the plankton bulked twenty to forty times as large as it did in the winter, the augmentation being most pronounced toward the outer edge of the continental shelf where the salpae were especially abundant. Early in October the haul volumes were of about the same size as in May, but a decline appeared by the middle of October in 1937 and at the end of that month in 1938 and the population presumably continued to shrink until the following January. The magnitude of this seasonal increase in volume appears to be considerably greater than that reported by Bigelow and Sears (1939) for even their best plankton years, but the contrast is largely due to the fact that our values at the beginning of the year are lower. Our summer volumes generally did not attain the size of those found by the earlier workers. In the present investigation the volumes of the crustacean plankton (Table II) followed much the same course as did the total plankton, since smallest values were taken in the winter and largest values during July and August. It is of interest to note that the crustacean element was still at a low ebb in April, and that in 1938 a period of scarcity occurred early in October followed by increased volumes at the end of the month, whereas in 1937 no prominent decline occurred until the middle of October.

In the slope water area little, if any, information existed previously on seasonal variation in richness. From our present data we may observe that although the average values for the total plankton did not vary over as great a range as was the case in the coastal area, and although the fluctuations were irregular, nevertheless, the hauls of smallest volume occurred during the colder part of the year and values as much as ten times greater were found in May and October. In the case of the crustacean plankton, however, the largest volume of the year was less than four times the smallest volume and both high and low values occurred at all seasons. Cognizance should also be taken of the fact that the average volumes of both the total plankton and of the crustacea differed widely in January, 1938 from those found in January, 1939 and a similar lack of agreement occurred between the values of early October, 1937 and 1938.

At our station in the Sargasso Sea there is even less evidence of a seasonal fluctuation in the richness of the plankton. As far as the total plankton is concerned, the largest average volumes were obtained in January, 1939, in April and in May–June, but volumes of almost equal magnitude were taken in September and October. Furthermore, since the individual hauls vary so greatly in size, we probably would not be justified in designating any one season as a period of increased plankton abundance. The same conclusion applies to the crustacea since the volume of this fraction of the plankton also varied irregularly, and since the highest value recorded was only about four times the lowest. It thus appears that among the three areas investigated indication of a significant seasonal change in richness for the total plankton was found only in the coastal and slope water zones and for the crustacean plankton only in the coastal area. Especially noteworthy was the occurrence of seasonal differences of greater magnitude than was suspected heretofore.

Regional Distribution

If the plankton volumes for all seasons are now combined, a basis is formed upon which a rough quantitative comparison may be made for the first time of the average differences in the richness of the several areas. This operation yields a grand average of 194 cc. for the volume of the zoöplankton of all types taken with the 75 cm. scrim net in the standard 30-minute haul in the coastal area. This figure is nearly four times as great as the average value of 52 cc. for the slope water area, which in turn is about four times the magnitude of the grand average of 12 cc. for our station in the Sargasso Sea (Table I). The average volumes for the crustacean plankton were 50 cc., 18 cc., and 7 cc. for the coastal area, the slope water area and the Sargasso Sea respectively (Table II). The abundance of the crustacea thus exhibited the same downward trend in passing offshore as was observed for the total plankton although the differences were only 2 or 3-fold in this case. Since we cannot yet make an adequate statement concerning the growth rate of the animals considered here, or rate of replenishment of the populations, no general conclusion can be drawn concerning the " productivity " of these areas in a broader sense. However, the present investigation does clearly indicate the existence in the coastal area of a " standing crop" which, on the average, significantly exceeds that characterizing the water farther offshore.

VARIATION IN ABUNDANCE OF CALANUS AND CENTROPAGES

The foregoing study of the variations in volume of plankton in the three oceanic areas under consideration has been supplemented by a numerical analysis of the fluctuations in two individual species of prominence—the copepods, *Calanus finmarchicus* and *Centropages typicus.* The variations in the abundance of Calanus are presented graphically in Fig. 7, where all stages which were caught by the net (copepodid stages IV and V and adults) have been added together and the shallow haul and deep haul (weighted) have been combined for each station.

It is clear from the graph that by far the major portion of the *Calanus* population is to be found in the coastal area, although a small number of this species was taken at Stations 6 and 7. No *Calanus finmarchicus* were found at Station 9 in the Sargasso Sea. The fact that the richest hauls were made at Stations 2, 3 and 4 with only moderate numbers at Station 1 is consistent with the distributional picture for this species presented by Bigelow and Sears (1939, Fig. 23). *Calanus* reached its greatest abundance during April, June, and July in the present survey as was also the case for the average of the years 1929–32 in the northern half of the area studied by Bigelow and Sears. At the station in the western entrance to Vineyard Sound occupied by Clarke and Zinn (1937), *Calanus* was similarly found to be most numerous from April to August. It therefore seems well established that the spring and summer months are the seasons at which *Calanus* comes into prominence as a member of the plankton community in this region.

The subdivision of the *Calanus* population into its sex and age groups at the stations in the coastal area throughout the year may be scrutinized in Fig. 8. The histograms here presented show that the great variability which characterized both the volumes and the species composition of the hauls also extends to the subdivisions within a single type of animal. Not only do the sex and age groups differ among the several stations on the same cruise, but in addition we find variations from year to year. Nevertheless, certain generalities appear. Copepodid stage V was the most numerous group for the investigation as a whole and particularly so during the autumn and winter months, but stage IV became prominent in May and June and in September. The occurrence of the adult females, which were the next most abundant, was rather irregular although there was some indication that this group was more usual near shore than at Stations 3 and 4. The fact that out of the total number

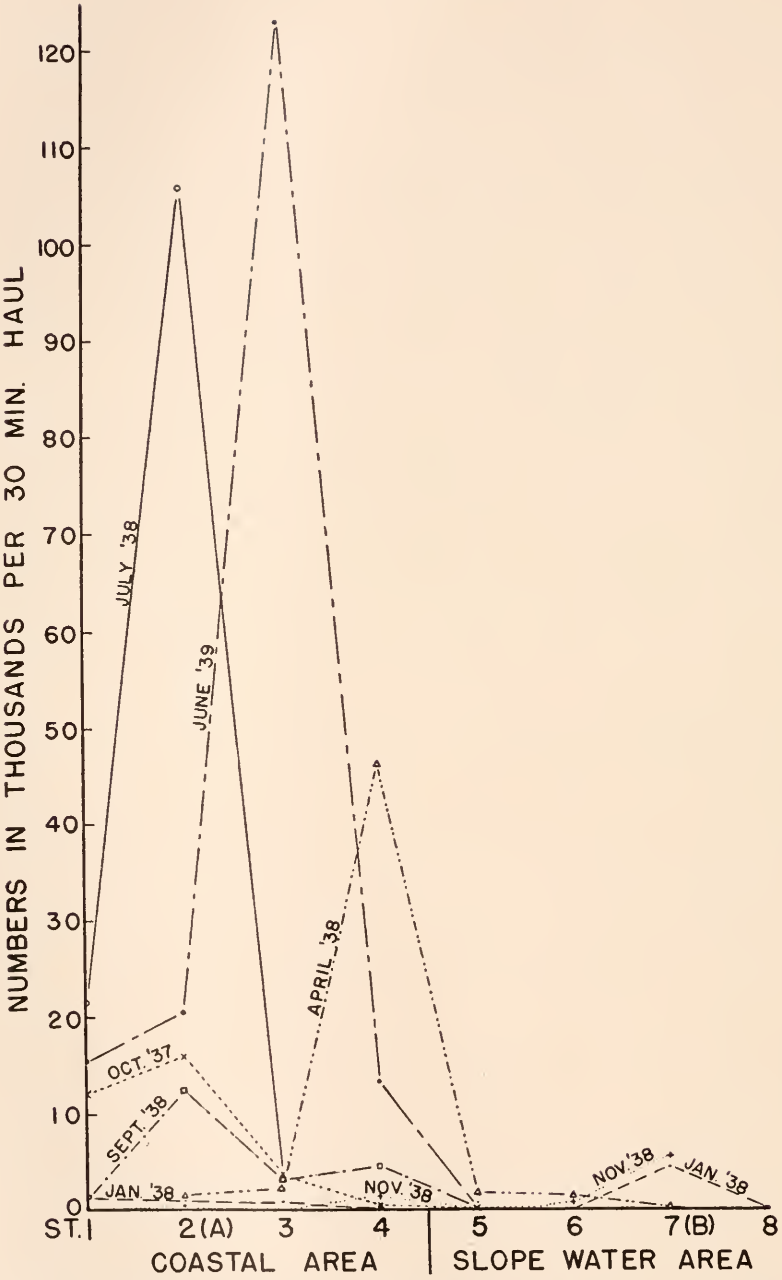

Fig. 7. *Variation in abundance of Calanus finmarchicus (all stages) throughout the year.* Shallow haul and deep haul (weighted) have been combined. Numbers of animals on cruise of Sept. 29–Oct. 2, 1938, too low to appear in graph. On cruise of Jan. 5–9, 1939, *Calanus* taken at Station 5 only.

of hauls adult males of *Calanus* were found only in January and in May and June agrees very closely with the observations at the station occupied several years earlier by Clarke and Zinn (1937). These investigators reported that *Calanus* had two breeding periods during the year, one resulting in a short-lived spring generation,[5] and the other in a long-

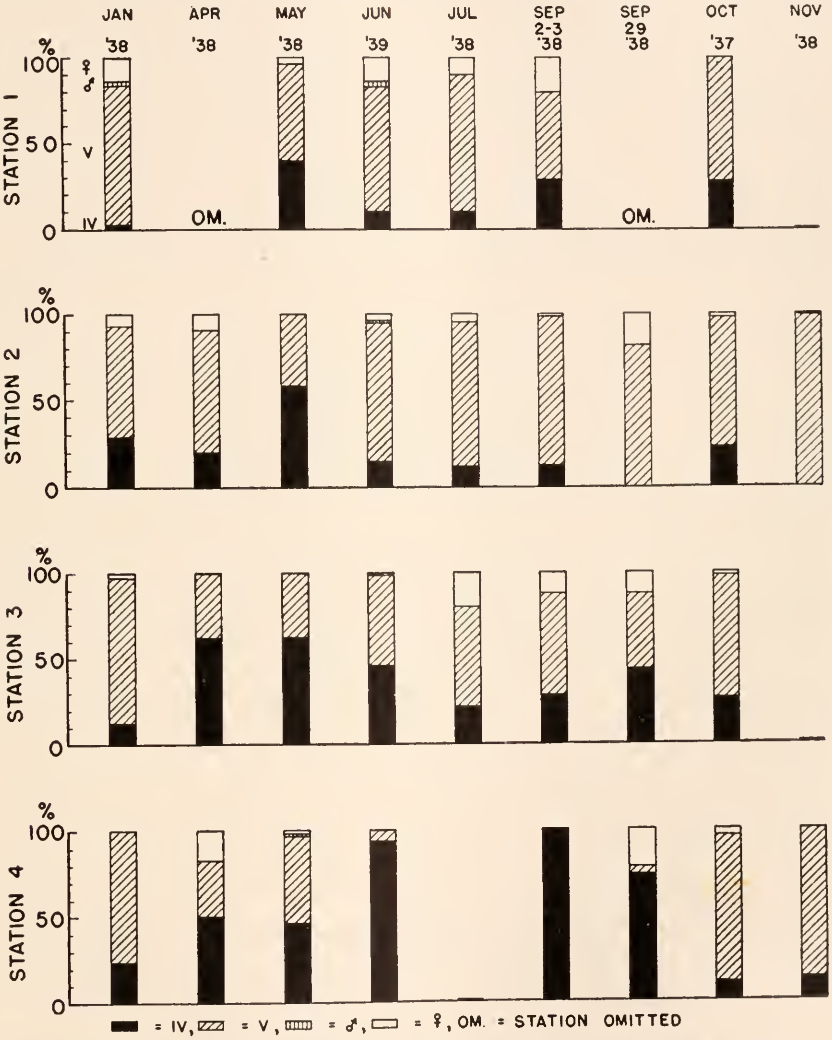

FIG. 8. *Percentage age and sex distribution of Calanus finmarchicus throughout the year* (coastal area only). On cruise of Jan. 5–9, 1939, no *Calanus* taken in this area.

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[5] The conclusions of Clarke and Zinn (1937) have been erroneously stated by Bigelow and Sears as "*two* shorter-lived generations during the spring, followed by a longer one in summer."

lived generation spanning the balance of the year. They also found that the appearance of the adult males presaged the commencement of breeding activity in this species. Consequently, the deduction seems justified that two generations of *Calanus* similarly occur each year over the whole of that part of the coastal area covered by the present investigation.

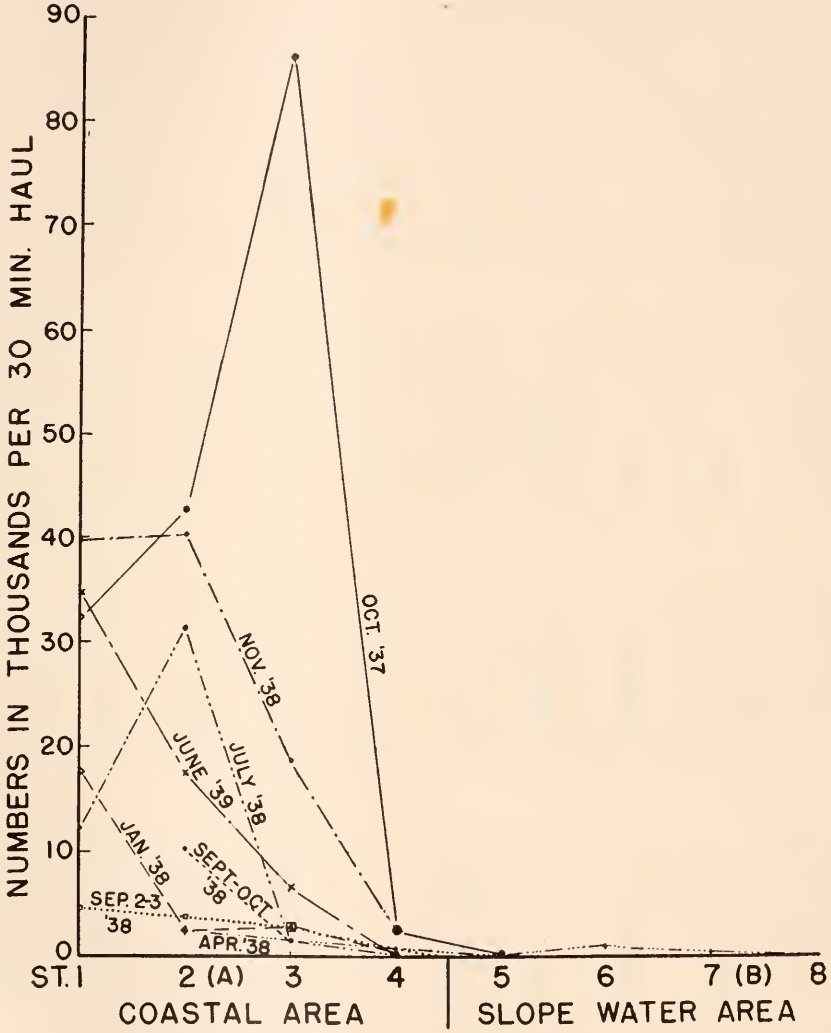

FIG. 9. *Variation in abundance of Centropages typicus (all stages) throughout the year.* Shallow haul and deep haul (weighted) at each station have been combined. On cruise of Jan. 5–9, 1939, *Centropages* taken at Station 3 only.

The horizontal distribution and seasonal changes in *Centropages typicus* are similarly presented in Fig. 9, in which the numbers of adult males and females and of immature specimens have been added together, and shallow and deep hauls combined. The graph shows that this species is restricted to the coastal area even more completely than was *Calanus*. Moreover, *Centropages* was never really abundant at the outer edge of this area (Station 4) and large numbers were frequently encountered near shore (Station 1). The population of this copepod was generally at a low ebb during the winter and spring months, but catches of greatly

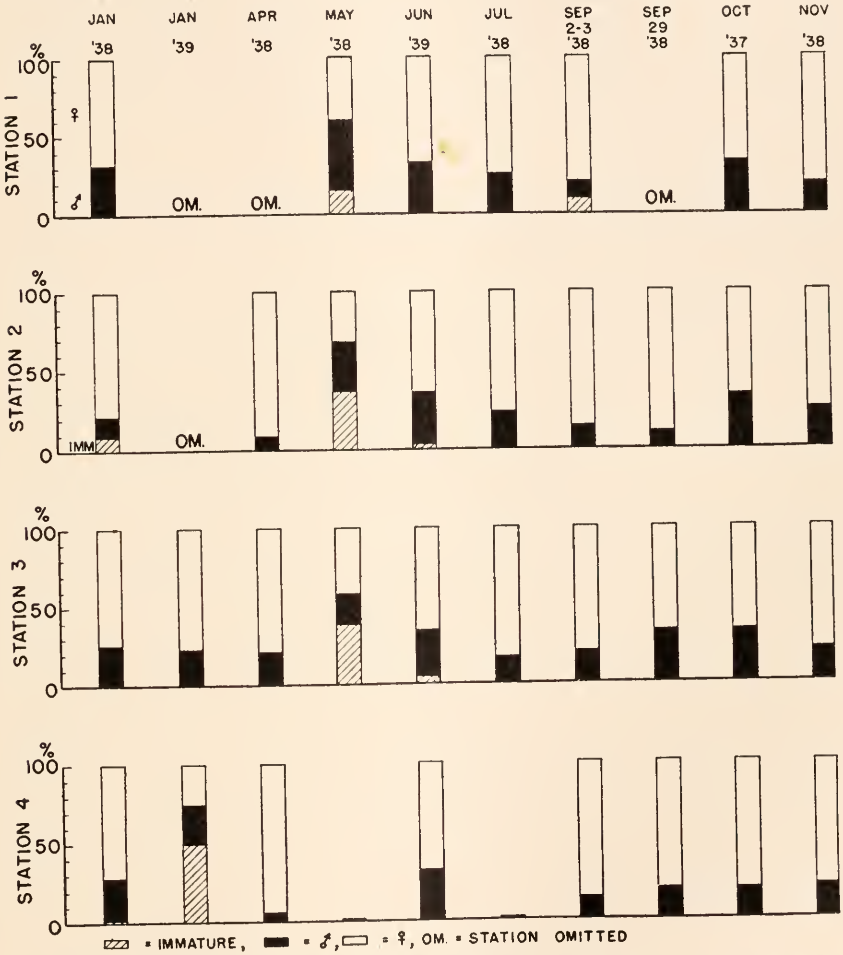

FIG. 10. *Percentage age and sex distribution of Centropages typicus throughout the year.* (Coastal area only.)

increased size were taken from June to November. The largest hauls of the series were made early in October, 1937 but numbers were relatively small during the same period in 1938, although sizeable catches were taken in November of that year. Yet, in spite of the fact that great irregularity is encountered here as elsewhere, we may conclude that in general *Centropages* reaches its peak of abundance during the summer at a period somewhat later than that typical for *Calanus,* and that it may continue to be plentiful well into the autumn. Our data, therefore, confirm the earlier deductions of Clarke and Zinn (1937) and of Bigelow and Sears (1939).

Both male and female adults of *Centropages* were well represented in our catches in the coastal area throughout the year in contrast to the situation with *Calanus* (Fig. 10). The females were more numerous than the males in almost every case but the males most nearly reached parity in May and June and again in September and October. The immature specimens were probably not taken in their true proportions by the relatively coarse scrim net, but if abundant in the water, they were represented qualitatively in the catches. A considerable percentage of immature *Centropages* was taken by the plankton samplers with No. 2 silk mesh in May, 1938 and by the scrim nets in January, 1939. Occurrences of smaller proportions were observed in January, 1938, June, 1939 and September, 1938. Clarke and Zinn (1937) report the immature stages of this copepod to have been particularly abundant in the summer months at their station. Bigelow and Sears (1939), however, did not distinguish between the immature and the adult specimens of this species. Our information is thus too scanty to determine the time of breeding for *Centropages,* but the fact that young individuals were encountered at every season except the autumn strongly suggests that more than one breeding period and probably more than one generation occur during each year. However, since the majority of *Centropages* evidently do not pass the autumn and winter in a late copepodid stage, it appears probable that the life cycle of this species differs markedly from that of *Calanus.*

DISCUSSION

The first impression which we derive from the foregoing data is one of variability. We have found that plankton hauls repeated within a short period at the same station or at nearby positions differed widely not only as to total quantity but also as to composition. Furthermore, from season to season the plankton oscillated with an amplitude which varied according to the location and which in certain cases was as great as 20- to 40-fold. In addition, both the type of plankton and its abundance in any area were found to differ in the same month according to the year. It appears, then, that any plankton study in an oceanic region of this type must be prepared to deal with time and space fluctuations on both a small and a large scale. This investigation has shown that no single station will serve as an adequate index for any of the areas under present consideration—a conclusion which was suspected from plankton work elsewhere. Also, it has become clear that a network of stations repeated throughout the year for several years will be necessary before the causes underlying these fluctuations can be ascertained.

As a step in this direction, the findings of the present investigation may be compared with and added to the results of the earlier explorations in these waters which have been thoroughly analyzed by Bigelow and Sears (1939). Unfortunately almost all the quantitative plankton work in this part of the Atlantic has been confined to the waters overlying the continental shelf. The whole of the area dealt with by Bigelow and Sears corresponds to only one of the zones crossed by our cruises—namely the coastal area. Nevertheless, since this region is the most changeable and also the most important from the point of view of the commercial fisheries, a comparison is highly desirable.

The location of the " Montauk Section," as designated by Bigelow and Sears, corresponds most closely to the position of our Stations 1 to 4. The volume of the total zoöplankton taken at stations on the Montauk Section during the years 1929–32 ranged from 3 to 1,880 cc., but out of a total of about 60 hauls only five hauls were less than 100 cc. and 17 hauls were greater than 500 cc. in volume. These figures contrast rather sharply with those obtained in the present investigation. Among our 58 hauls in the coastal area only 5 were more voluminous than 500 cc. and 31 contained less than 100 cc. The volumes of hauls in the present survey were generally inferior even to those of the years 1929 and 1932 which were reported to be years of plankton scarcity. The discrepancy may be explained to some extent by the difference in the type of net employed, for the plankton measured by Bigelow and Sears was taken with a net 1 meter in diameter, the top of which was made of No. 0 silk (15 strands/cm.) and the tail of No. 2 silk (21 strands/cm.). In addition to the fact that this finer mesh silk would tend to catch more of the smaller organisms than the scrim employed by us, it may well be that the filtering efficiency of the silk is superior to that of the scrim, which was estimated by Clarke and Zinn (1937) to be as low as 20 per cent. However, the effect of the larger net and finer mesh is offset to some extent by the fact that Bigelow and Sears reduced their data to a standard haul of 20 minutes instead of the 30 minutes employed in the present study. The combined effect of the differences in net size and in duration of haul would yield a theoretical factor of .84 to be applied to the volumes reported by the earlier workers, but the allowance which should be made for other differences in method is not known. Although the discrepancy is very likely not as great as would at first appear, nevertheless, everything considered, it seems probable that the plankton was really less abundant during the period of the present survey than it was during any of the years 1929–32.

A much more precise measure of the actual abundance of the plankton may be obtained from the hauls made with the 5-inch plankton samplers since these instruments are equipped with calibrated flow meters. Unfortunately, the samplers were not developed early enough to be used for the whole investigation, but it is of some value to examine such hauls as we have for their order of magnitude. Although the samplers are extremely useful in coastal regions, they are less satisfactory (in their present design) for work in the areas farther offshore since the scarcity of the plankton causes the catches to be inconveniently small. Even though these hauls were frequently too small to measure accurately, nevertheless, the upper limit of the richness of the population may be obtained from them. The abundance of the plankton as measured by the plankton sampler is summarized below:

	Coastal Area (Stations 1–4)	Off-shore Areas (Stations 5–9)
Total number of hauls	67	71
Number < 0.1 cc./m.^{3}	15	51
Number > 1.0 cc./m.^{3}	15	5
Average of cases 1.0–0.1 cc./m.^{3}	0.54 cc./m.^{3} (37 cases)	0.40 cc./m.^{3} (15 cases)
Maximum abundance	15.5 cc./m.^{3}	3.5 cc./m.^{3}

The foregoing data point once again to the characteristically greater richness of the coastal area as compared with the offshore areas. Beyond the edge of the continental shelf the concentration of plankton was less than 0.1 cc./m.^{3} in about 75 per cent of the cases whereas in the coastal area more than 75 per cent of the hauls indicated an abundance greater than 0.1 cc./m.^{3}. All the catches greater than 2.0 cc./m.^{3} in the coastal area and greater than 1.0 cc./m.^{3} in the offshore areas were dominated by salpae. Since in the coastal area an equal number of very large and very small hauls were taken, we may regard the majority of catches of intermediate size (1.0–0.1 cc./m.^{3}) as typical of this region. The average of the cases which fell in this category (almost all of which occurred in the warmer half of the year) is 0.54 cc./m.^{3} and may be compared with the estimate of the absolute abundance of the plankton in this area made by Bigelow and Sears and based on hauls with 1-meter nets. These investigators reported averages of "about 0.5–0.8 cc. per cubic meter at the season of maximum production." These two estimates, reached by very different methods, agree so closely that we are led to place considerable reliance on the order of magnitude of the values obtained as an index of the actual richness of plankton in this coastal region (cf. Clarke, 1939*b*).[6] The fact that our average value is found to be at the lower limit of the range given by Bigelow and Sears adds support to the conclusion reached above that the period of the present investigation was one of relative plankton scarcity.

A further evidence that the plankton under consideration here differed significantly from that present during the period of the earlier investigation is furnished by the unusual abundance of salpae which we encountered. Bigelow and Sears reported that salpae, medusae and ctenophores ordinarily accounted for only a small percentage of the entire catch but that a maximum value of 22 per cent for the area as a whole was found in June, 1932. In our hauls, however, the total plankton for the four coastal stations combined amounted to more than twice the volume of the crustacean plankton on 6 out of the 10 cruises and on certain occasions it was several times more abundant. The inference is that during the period from October, 1937 to June, 1939 in our coastal area the plankton as a whole was relatively scarce and that the crustacea were particularly depleted, while the other elements—especially the salpae—were unusually abundant. The recurrence of such important differences in both the quantity and the complexion of the plankton in the waters over the continental shelf and their undoubted significance in the ecology of the region is further elaborated elsewhere (Sears and Clarke, 1940).

As yet we have no adequate information on the possible occurrence of annual variations in the plankton in the slope water area or in the regions farther offshore. Further quantitative investigation is demanded. Nevertheless, it is doubtful whether the plankton beyond the edge of the continental shelf ever reaches the proportions of that within the coastal area. What factor essentially underlies the differences in the richness of the standing crop in the three areas remains to be worked out. The characteristic differences in depth and the seasonal changes in temperature, which have been described above, determine what portion of the whole water column can be effectively stirred and thus bring about a regeneration of nutrients and a renewed impetus to biological production. But the details of these events are not yet adequately known and much remains to be learned of the rates of reproduction and growth of the dominant organisms in these areas before the characteristic annual productivity of each can be ascertained. Nevertheless, the present observations make it clear that a distinct difference in the richness of the plankton of the three areas existed during the period of this investigation and since the indications are that this period was one of relative scarcity for the coastal area, we may suspect that the ratio of plankton abundance in waters over the continental shelf to that farther offshore is in general even greater than was found in the present case.

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[6] The agreement also adds support to the conclusion of Clarke and Bumpus (1939) and of Winsor and Clarke (1940) that a plankton net only 5 inches in diameter is as reliable as a meter net for a quantitative measure of the plankton.

Summary

1. The abundance of the zoöplankton in the coastal water, the slope water, and the Sargasso Sea at stations between Montauk Point, N. Y., and Bermuda was investigated over a period of a year and a half by means of shallow and deep hauls made chiefly with 75 cm. scrim nets. Analysis of the plankton by volume measurement and by enumeration revealed that wide variations both in the quantity and in the composition of the hauls occurred within short periods of time and within relatively short distances.

2. A noticeable diurnal migration of the crustacean plankton was detected in the coastal water and particularly in the slope water, although the vertical distribution of the plankton as a whole was not significantly affected by diurnal movement. When all observations are combined, the surface layers do not appear to have been generally richer than the deeper strata except in the case of the Sargasso Sea station.

3. An unexpectedly great seasonal difference in the abundance of the plankton was revealed, the volume of the catches in the warm half of the year being as much as 20 times or 40 times greater than in the winter in the coastal area and as much as 10 times greater in the slope water area, but no significant seasonal change was indicated in the Sargasso region.

4. When the shallow and deep hauls throughout the year are combined, the investigation as a whole shows that the average volume of the plankton was about 4 times greater in the coastal area than in the slope water area and that the latter was about 4 times greater than at the Sargasso Sea station. Differences in the same direction but of smaller magnitude were found for the crustacean plankton.

5. Individual study of *Calanus finmarchicus* and of *Centropages typicus* showed that the abundance of these copepods, which were confined to the coastal area, fluctuated widely, the former being most numerous in spring and early summer and the latter in late summer and autumn.

6. The actual richness of the plankton in the coastal area as measured by the plankton sampler fell within the approximate range observed by earlier investigators, but all indications point to the period of the present study as one of relative scarcity. In addition, the proportion of the crustacea in the hauls was unusually low (less than half the total plankton in 6 out of 10 cruises) and the number of salpae was unusually high. The superiority in richness of the coastal area over the offshore areas probably is generally even greater than that found in the present case.

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OXYGEN CONSUMPTION AND ITS INHIBITION IN THE DEVELOPMENT OF FUNDULUS AND VARIOUS PELAGIC FISH EGGS

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INTRODUCTION

Loeb (1895) has shown that during the first few days after fertilization the egg of *Fundulus heteroclitus* is capable of extensive development under anaerobic conditions in contrast to the immediate suppression produced in the developing *Ctenolabrus* egg. Anaerobic development in amphibian eggs has also been observed by earlier workers and has recently been confirmed by Brachet (1934) in *Rana fusca* and *Discoglossus* eggs with the use of HCN as a respiratory inhibitor. Cyanide, like other hemochromogen-binding reagents such as NaN_3 and CO, acts as a respiratory inhibitor by combining in a specific, reversible manner with respiratory catalysts like cytochrome-oxidase (Keilin, 1933). The activity of these three reagents has already been used to advantage on the *Fundulus* egg for demonstrating the presence of cytochrome-oxidase in the chain of reactions which control the frequency of the embryonic heart-beat (Fisher and Cameron, 1938; Armstrong and Fisher, 1939; Fisher and Ohnell, 1938). If the respiratory systems of both *Fundulus* and *Ctenolabrus* eggs are also sensitive to cyanide and azide, the effect of these substances on the development of these eggs ought to be analogous to the effect of anaerobiosis. Therefore it would be of interest to apply a similar sort of analysis as that of Fisher and co-workers to the development of the embryos of these fishes and to compare the results obtained with those of Loeb.

Various studies have been made of the respiration of the *Fundulus* egg during early development. Boyd (1928) has reported a striking but temporary increase in respiration shortly after fertilization. Hyman (1921) has found that there are fluctuations in respiratory rate during *Fundulus* development, and the studies of Trifonova (1937) have shown similar changes in the perch. However, such fluctuations are not found in amphibian eggs where increases in respiratory rate have been shown to occur continuously by Brachet (1934), Atlas (1938), and Steffanelli (1938). Both Hyman and Trifonova have attempted to

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relate these changes in the fish embryos to specific morphological processes.

A careful study of the respiration of the *Fundulus* egg throughout development to hatching has been made by Amberson and Armstrong (1933); but since their method of measurement did not allow for the detection of possible fluctuations in respiratory rates within daily intervals, it would seem of value to re-study the same problem during early development by another method.

This paper deals with the two problems, (1) the respiration of the *Fundulus* egg during the first two and one-half days of development and its sensitivity to NaCN, and (2) the comparison of the development of the *Fundulus* egg with that of several pelagic, fish eggs in various concentrations of NaN_3 and NaCN.

Materials and Methods

Fertilized eggs of *Fundulus heteroclitus* and of the cunner, *Tautogolabrus adspersus* (*Ctenolabrus*), were obtained by stripping sexually mature females and males. The eggs of mackerel, *Scomber scombrus,* and scup, *Stenotomus chrysops,* were provided by the U. S. Bureau of Fisheries within a few hours after stripping. All eggs were allowed to develop at laboratory temperatures (20–25° C.).

For the measurements of respiration Fenn volumetric micro-respirometers were used. The apparatus had capillary volumes of about 0.8 cu. mm. and 2.0 cu. mm. per cm. of length. Apparatus constants calculated according to the Fenn (1928) equation were respectively about 0.9 and 2.4 cu. mm. per cm. of capillary length. These sensitivities were obtained by modifying the sizes of the respirometer vessels so that the ratio of control to experimental gas spaces was about 4/1 (27 ml. to 7 ml.). The respirometers were shaken in a water-bath maintained at 22° C. at the rate of 110 times per minute through an arc whose chord was 7.5 cm. The carbon dioxide produced was absorbed by molar KOH in the side-arm of the experimental vessel. The volume of the eggs and sea water was always brought to 1 ml.

The respiration measurements were made on fertilized eggs immediately after they had been washed in sea water to remove excess sperm. The eggs were counted at the end of each experiment and the data were rejected if more than 10 per cent of the eggs were abnormal or unfertilized. In experiments on later embryological stages, however, only normal-appearing eggs were used. Between 50 to 100 eggs were used in each experimental vessel. Eggs once used for a cyanide experiment were discarded. The determinations of the cyanide sensitivity of the respiration were all carried out in M/1,000 NaCN in sea water. The NaCN solutions were adjusted to the pH of sea water with HCl. In these determinations the KOH of the side-arm was replaced by Krebs' (1935) KCN–KOH mixture. No correction was made for the use of this mixture with sea water and it is possible that the total concentration of cyanide in the solution surrounding the eggs was greater than M/1,000. In order to determine the cyanide-insensitive respiration accurately it was necessary to use about twice as many eggs as in the experiments with unpoisoned controls. All respiratory rates are given in cu. mm. O_2/egg/hour.

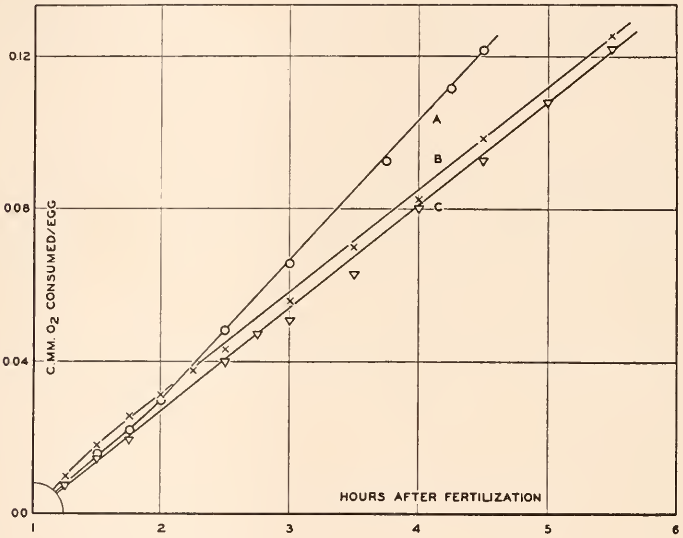

Fig. 1. Oxygen consumption of fertilized *Fundulus* eggs. For curves *A, B,* and *C,* respectively 75, 58, and 94 eggs were used.

To determine the effects of NaCN and NaN_3 on the development of *Fundulus,* 50 to 100 fertilized eggs were placed in 150-ml. Erlenmeyer flasks with 25 ml. of freshly prepared solution. The eggs were well covered; but the solution was shallow enough so that an adequate supply of oxygen was available. The solutions were changed three times daily. In the experiments with pelagic eggs several milliliters of a concentrated suspension of fertilized eggs were added to each flask with 50 ml. of sea water solution. Since the eggs float on the surface, there is no problem of oxygen diffusion through the solution. With these eggs the rapidity of development obviated the necessity of prolonged observation and therefore no change of solution was made. The

NaCN solutions were made up in sea water and brought back to the pH of sea water with HCl. As the pH of the NaN_3-sea water solutions is lowered, the concentration of HN_3 increases resulting in higher concentrations of azide within the egg. Thus the inhibitory effect of NaN_3 solutions is increased by lowering the pH (Keilin, 1936; Armstrong and Fisher, 1939). For this reason the NaN_3 solutions were made up with sea water which had been acidified with HCl and equilibrated by passing a strong stream of air through it for several hours. In a few experiments sea water buffered with M/100 phosphate buffer was used; but this method is complicated by the deposition of insoluble phosphate salts in experiments of duration longer than about 12 hours. The pH values were determined with the glass electrode.

In recording the stages of development of the *Fundulus* eggs in these respiratory-inhibitor experiments fifty or more eggs from each solution were examined with the aid of a binocular dissecting microscope. The stages were designated according to Oppenheimer (1937), and then the eggs were returned to the original solutions. In the case of the pelagic eggs a sample portion of those still floating was removed from the solutions and the stage found representative of the majority of at least 30 living eggs was recorded. This presented no difficulty since the pelagic eggs no longer float after dying.

EXPERIMENTAL RESULTS

The Respiration of the Fundulus Egg

The respiratory rate within an hour after fertilization appears to have a more or less constant value as shown in Fig. 1. In these experiments the rates of oxygen consumption were far above the limit of sensitivity of the apparatus. They involved indicator drop movements of 1.5 to 3 cm. per hour. There is no evidence for a significant rise in respiratory rate to a maximum at about 90 minutes after fertilization with a subsequent decline as expected from Boyd's results (Table I).

In Fig. 2 are plotted a number of individual experiments carried out on eggs at various times during the first two days of development. The complete graph of all the determinations of respiratory rates of unpoisoned eggs is shown in the upper curve of Fig. 3. The individual points in the upper curve of Fig. 3 are the rates per egg per hour calculated for periods of 1–2 hours duration. No more than two points are taken from a single experiment and these are separated by an interval of at least four hours.

The curve which is drawn through these points has been fitted to the averages of all the rates within successive three-hour periods. No emphasis is to be placed on the exact nature of the curve. The character of the break which is shown cannot be accurately determined from the data at hand. However, it is clear that after about 15 hours of development a change occurs in the rate of increase of oxygen consumption.

Figure 2 shows that the rate of oxygen uptake increases with advance in development of the fertilized egg. This is also brought out in Fig. 3 where the actual rates of respiration per egg can be seen to rise with the increase in time after fertilization. Further, in the individual experiments of Fig. 2 and in the composite of results in Fig. 3 the rise in the rate of oxygen consumption in the period between six and fifteen hours after fertilization is more rapid than it is before six or after fifteen hours. Moreover, during the six to fifteen-hour period the embryo is advancing from the very early high blastula to the flat blastula stage.[1] The morphological changes which do occur during this segmentation period involve a large increase in cell number, and it is probable that this is directly associated with the rapid increase in respiratory rate. There is no evidence, from the data presented in Figs. 2 and 3, of any major fluctuations in respiratory rate which might possibly be

TABLE I

Rate of oxygen consumption of fertilized *Fundulus* eggs. Calculations are for successive half-hour periods after fertilization. Rates are given as cu. mm. O_2 consumed/egg/hour.

No of Eggs	Percentage Abnormal	Hours after Fertilization								
		1–1½	1½–2	2–2½	2½–3	3–3½	3½–4	4–4½	4½–5	5–5½
78	1	—	—	.029	.035	.038	.037	.044	.054	—
108	9	—	—	—	—	—	.029	.022	.029	.026
94	5	.029	.026	.026	.022	.032	.036	.027	.032	.026
75	4	.032	.028	.037	.035	—	.036	.039	—	—
66	3	—	.033	.035	.030	—	.031	.037	—	—
58	9	.036	.027	.024	.025	.028	.025	.032	.027	.027
51	0	.036	.027	.023	.023	.036	.026	.025	.040	.040
Averages		.033	.028	.029	.028	.034	.031	.032	.036	.030

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[1] The description of the normal stages of *Fundulus* is taken from Oppenheimer (1937).

correlated with definite morphological events such as the beginning of gastrulation. On the contrary, there is a continuous, if not constant, increase in respiratory rate with advance in embryological differentiation.

Considering the differences in the two methods of measurement there is fairly good agreement between the results presented in Fig. 3 for the first two days after fertilization and those of Amberson and Armstrong. They found rates between 0.7 to 0.9 and 1.9 to 2.2 ml./day/1,000 eggs for the first and second day, respectively. By appropriate change of units and by summation of graphically determined values for successive three-hour periods the present data give values of about 1.2 and 2.6 ml./day/1,000 eggs.

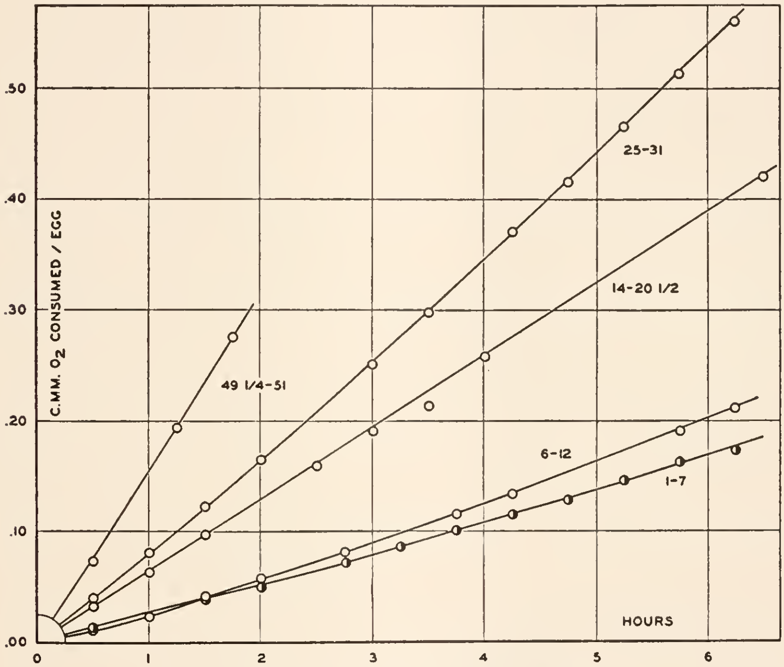

FIG. 2. Oxygen consumption of developing *Fundulus* eggs. Figures adjacent to curve indicate time in hours after fertilization.

The Cyanide Sensitivity of Fundulus Respiration

During the first six hours after fertilization the respiration of *Fundulus* eggs in M/1,000 NaCN solutions was found to be 32 per cent of the average normal respiration. In order to ascertain the cyanide-stable respiration of later stages, eggs were allowed to develop in normal sea water until the desired stages were reached. They were then placed in the cyanide medium and their oxygen consumption determined for a period of at least two hours. The maximum respiratory inhibition was always attained within less than an hour after transfer to the M/1,000 NaCN. The results of these experiments are shown in the lower curve of Fig. 3. If the two curves of Fig. 3 are compared, it becomes evident that the cyanide-resistant respiration increases slightly in rate within the first day and then remains constant throughout the next day and a half. Actually, in two determinations at 98 hours after fertilization the cyanide-stable respiration had still remained unchanged, viz., 0.020 and 0.022 cu. mm./egg/hour, as compared to .019 cu. mm./egg/hour at about 62 hours. The increased respiration during the first four days of development may thus be seen to be almost exclusively the result of a cyanide-sensitive respiratory system. That this is the cytochrome-oxidase system is expected from the work of Fisher and co-workers.

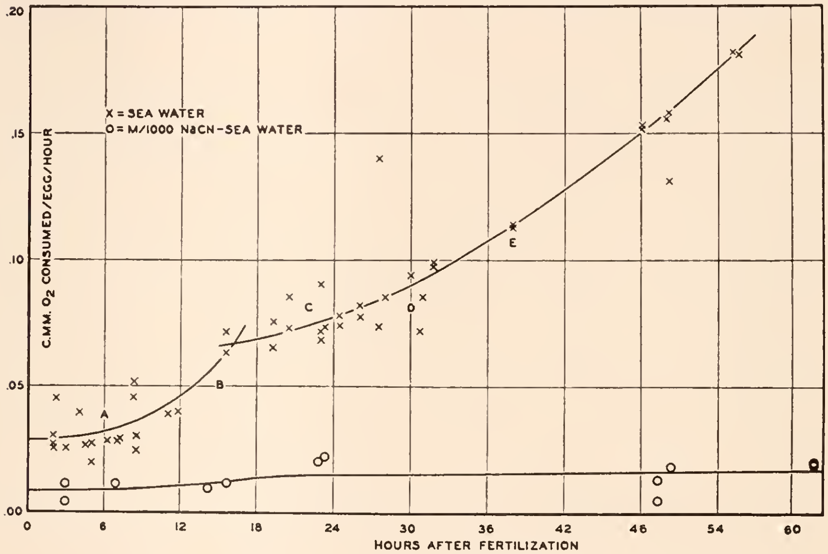

FIG. 3. Rate of oxygen consumption during the first two and one-half days of development of *Fundulus* in untreated sea water and in M/1,000 NaCN. *A*, early high blastula; *B*, flat blastula; *C*, early gastrula; *D*, late gastrula; *E*, appearance of optic vesicles. For the determination of the cyanide-sensitivity of different stages of development eggs were raised in normal sea water to the desired stage, transferred to cyanide sea water in the respirometers, and rates determined over two-hour periods.

The Effect of Respiratory Inhibitors on the Development of Fundulus

So far it has been shown that the inhibition of respiration of the *Fundulus* egg in M/1,000 NaCN is maximal within an hour after transfer from normal sea water. It is of interest now to investigate the effect of NaCN-sea water solutions on the development of the egg. In contrast to the immediate effect produced by NaCN on their respiration, eggs placed in solutions of M/1,000 NaCN approximately one-half hour after fertilization and prior to the completion of the blastodermic cap, developed to late high and flat blastula stages before coming to a complete halt. The rate of development of such eggs is not much slower than the controls. In one experiment at 20 hours after fertilization, eggs in M/1,000 NaCN were found to be in the late high or flat blastula stages (9 and 10 of Oppenheimer). The sea water controls were mostly in the flat blastula stage with only about one-fifth already expanding blastulae (Stage 11). This is a difference of less than six hours of normal development. It is very probable that the eggs in M/1,000 NaCN had reached a state of complete developmental inhibition several hours before the time of observation which might account for most of the difference. Subsequent observations on the same inhibited eggs at about 33 and 48 hours after fertilization revealed no further development. Thus it is clear that eggs in M/1,000 NaCN continue to develop from shortly after fertilization to the late blastula stages before they are completely inhibited. Further, the rate of this development in M/1,000 cyanide is not much slower than that of control eggs in normal sea water.

The effects of other concentrations of NaCN on the development of eggs soon after fertilization are shown in the results of a similar experiment (Table II). At 22 hours the eggs in the most concentrated NaCN solution were in stages attained by untreated eggs about eight hours earlier. Twenty-four hours later the eggs in M/2,000 NaCN had developed no further than normal eggs do in about six hours. From then on the eggs in M/2,000 NaCN exhibited little, if any, significant development. Altogether, these eggs have undergone a total development equivalent to that found in untreated eggs in about 20 hours. By two days it is also seen that the eggs in M/2,000 NaCN have reached the maximum development possible. In M/8,000 and M/16,000 NaCN the eggs continue to develop beyond the two-day period but more slowly than the controls.

The experiments described above have shown that eggs, transferred to the higher concentrations of NaCN, M/1,000 or M/2,000, within a few hours after fertilization, are able to develop to the beginning of gastrulation. This might possibly indicate that at this stage there exists an increased sensitivity to the presence of high concentrations of NaCN. To test this possibility eggs were allowed to develop in normal sea water until the flat and expanding blastula stages (10 and 11) and then transferred to NaCN solutions. Such eggs in M/2,000 NaCN became middle gastrulae (Stage 13) before development was suppressed. This is equivalent to the amount of development occurring in normal eggs in about 10 hours. Correspondingly, 71 hours after fertilization eggs in M/4,000 NaCN were still in the stage of blastopore-closure (Stage 15); in M/8,000 NaCN embryos had developed hollow optic vesicles (Stage 17); but in the M/16,000 solution development was beyond the recorded stages. It is clear from such data that eggs which are already beginning to gastrulate are still capable of considerable development under conditions of reduced respiration. Furthermore, these eggs develop beyond stages at which suppression occurred when cyanide was used from the beginning of development. This indicates that there are no specific morphological stages at this period of development where sensitivity to

Table II

Development of *Fundulus* eggs in NaCN. Experiment started about three hours after fertilization when eggs were still in two-cell stage. Observations were made after 22, 48, and 71 hours.

Hours after Fertilization	Concentration of NaCN	Total No. of Eggs Observed	Percentage Abnormal or Dead	Oppenheimer Stages of Fundulus Egg								
				9	10	11	12	13	14	15	16	17
22	M/2000	67	13	21	37							
	M/4000	50	24	2	36							
	M/8000	62	39		1	37						
	M/16,000	74	39		1	43	1					
	Control *	65	32		1	21	22					
48	M/2000	65	37			38	3					
	M/4000	61	33				8	36				
	M/8000	62	45						2	26	6	
	M/16,000	68	40								14	27
71	M/2000	63	51			20	11					
	M/4000	65	35				41	1				
	M/8,000	76	42							2	19	23

* No further observations of control eggs are included in table since at the later times the control eggs had developed beyond the stages recorded in these experiments.

cyanide poisoning (i.e. to reduced cellular oxidations) is particularly evident. However, the older eggs are able to develop for a shorter duration of time, measured from the beginning of cyanide treatment, than are the younger eggs.

It is important to consider the possible relationship between suppression of development and death of eggs in the NaCN solutions. In Table II it will be noted that the percentage of eggs which are recorded as abnormal or dead increases during the course of the experiment. This might be interpreted as indicating that the suppression of development manifests itself in the death of eggs at a stage of development of increased sensitivity to the poison.

There are, however, several reasons for not accepting such an interpretation. In the first place, an inspection of Table II reveals that at 22 hours there is no obvious connection between the concentration of NaCN and the number of dead eggs. In fact, their number in the control is greater than in M/2,000 or M/4,000 NaCN. Further, eggs recorded as existing in arrested states of development are actually alive, as was tested by transferring them into normal sea water which removes the cyanide inhibition and allows development to continue. After several days in normal sea water, it was found that the percentage of developing eggs was the same as the percentage of those which had been considered in arrested states of development during NaCN treatment. Of all the eggs previously in M/2,000, M/4,000 and M/8,000 NaCN (Table II) respectively 46, 51, and 71 per cent were developing.[2]

There are additional objections to the interpretation that suppression of development is the result of the death of the eggs in stages of increased sensitivity to cyanide. One of these is the fact that the percentages of abnormal or dead eggs did not necessarily increase during the period of complete inhibition in every experiment (see Table III). Moreover, it has already been pointed out that during the period of development studied in this paper there is no evidence for an increased cyanide-sensitivity at any particular developmental stage.

The results with solutions of NaN_3 are essentially similar to those with NaCN (Table III). Results with eggs transferred to NaN_3 from normal sea water in the middle gastrula period (Stage 13) were also similar to those obtained with NaCN.

[2] Many of the eggs which develop further after the extended developmental arrests in the poison solutions show numerous abnormalities. This may be expected from any inhibitory treatment of the early embryonic stages of the *Fundulus* egg (Stockard, 1921). No analysis of these abnormalities was made since the primary interest here was whether the previously inhibited eggs were still capable of development.

The Effect of Respiratory Inhibitors on the Development of Pelagic Eggs

When experiments similar to those described above were carried out with some pelagic fish eggs, entirely different results were obtained. If, for instance, cunner eggs in the two-cell stage were transferred to M/1,000 NaCN solution, they did not develop beyond the formation of the second cleavage plane. This state was reached by them and by the control eggs within 20 minutes. Similarly (Table IV) in M/10,000 NaCN, eggs never developed beyond the stage reached by the controls in one-half hour. Moreover, late blastulae or early gastrulae, when treated with M/10,000 NaCN, never advanced beyond these stages.

Table III

Development of *Fundulus* eggs in NaN_3. At time of making up fresh solutions pH = 6.2–6.8. Experiment started about three hours after fertilization when eggs were in four- and eight-cell stages.

Hours after Fertilization	Concentration of NaN_3	Total No. of Eggs Observed	Percentage Abnormal or Dead	Oppenheimer Stages for Fundulus							
				9	10	11	12	13	14	15	16
47	M/500	50	40	11	19						
	M/1000	47	21		6	27	4				
	M/2000	41	20			8	25				
	M/5000	39	13				4	30			
	M/10,000	43	16						2	34	
70	M/500	51	41	7	20	3					
	M/1000	53	23		9	32					
	M/2000	42	26			8	23				
	M/5000	44	23					34			
	M/10,000	47	11						1	26	15

Further examination of Table IV reveals that in M/20,000 and M/40,000 NaCN the cunner eggs were able to proceed to later stages before complete inhibition resulted. However, the rate of their development to these stages was definitely less than that of the control eggs. A still different situation existed in M/80,000 and M/100,000 NaCN solutions where the eggs showed a decrease in rate of development only at a relatively later time and never were inhibited completely. As early as 4½ hours and 7 hours the eggs in M/80,000 appeared qualitatively as somewhat retarded late blastulae as compared with eggs in M/100,000 NaCN, or in the control flask. Such results are not to be interpreted as arising from the effect of decreased rates of penetration of NaCN from the less concentrated solutions. It has been shown above that eggs in the more dilute concentrations demonstrate the presence of cyanide internally by retardation in development for a considerable length of time before complete developmental arrest takes place. Furthermore, an increase in sensitivity to NaCN during development will not account for the inhibitions. Experiments show that eggs transferred from normal sea water to NaCN after having reached the late blastula stage are still capable of undergoing gastrulation for several hours in such concentrations as M/40,000.

TABLE IV

Development of cunner eggs in NaCN. Experiment started one-half hour after fertilization when eggs were still in stage of formation of blastodermic cap.

Concentration of NaCN	Hours after Addition of NaCN						
	½	1	1¾	4½	7	20	27
M/10,000	2 cells	2 cells	2 cells	disintegration			
M/20,000			4 cells	32–64 cells	disintegration		
M/40,000			8 cells	early high blastula	early high blastula	disintegration; early high blastula	
M/80,000			16 cells	late high blastula	late high blastula	2/3 gastrula	embryo 1/2 around yolk
M/100,000			16 cells	late high blastula	late high blastula	2/3 gastrula; closure of blastopore	embryo 2/3 around yolk
Control	2 cells	4 cells	16 cells	late high blastula	late high blastula	10 somites; embryo 1/2 around yolk	embryo 5/6 around yolk

Several hours after complete inhibition of development had occurred in these pelagic eggs disintegration became evident. The cell walls of early cleavage stages appeared to dissolve resulting in a completely undivided blastodermic cap which eventually became opaque. At the same time shrinkage of the yolk occurred and the eggs no longer floated at the surface of the solution. If later stages were attained before complete inhibition, an increasing disorganization and opacity of the embryonic tissues with yolk shrinkage and loss of buoyancy occurred. All these observations are in complete agreement with those that Loeb obtained on *Ctenolabrus* (cunner) under anaerobic conditions.

The results of experiments with NaN_3 on cunner eggs were similar to those obtained with NaCN. It is interesting to note that the concentrations of NaN_3 required to inhibit the development of the cunner eggs were about ten times higher than those treated with NaCN (Table IV). At present there is no available explanation for this observation.

In the earlier part of the summer it was possible to carry out a few experiments on mackerel eggs. The concentrations of NaCN used, ranging from M/200 to M/10,000, inhibited development equally rapidly. Eggs poisoned during the second cleavage proceeded to fourth cleavage while those in the third proceeded to the fifth cleavage. In both cases this was equivalent to the development occurring in the control eggs in one hour. Moreover, the experimental were somewhat slower than the control eggs in reaching the limit of their development. Eggs of later stages were equally sensitive.

When eggs in the second cleavage stage were transferred to unneutralized sea water, M/100 with respect to NaN_3, cleavage proceeded only to 32 cells before being completely inhibited. M/1,000 NaN_3 under similar conditions stopped development in the early gastrulation stages. By using sea water, buffered to lower pH (6.2–6.3) with phosphate salts, it was possible to inhibit the eggs of the 2-cell stage within one cleavage with M/1,000 NaN_3. Even lower concentrations produced complete inhibition.

In one experiment with scup eggs at two different stages NaN_3 was used in phosphate-buffered sea water. Beginning with the one-cell stage eggs in M/1,000 proceeded as far as 4 to 16 cells while in M/10,000 inhibition was not complete until the late high blastula stage. Scup eggs transferred to M/10,000 NaN_3 in the late high blastula stages proceeded only to middle gastrulae.

Discussion and Conclusions

Within one hour after fertilization the oxygen consumption of the *Fundulus* egg persists at a rate which tends to rise in a continuous, but not constant, fashion throughout early development. In agreement with Amberson and Armstrong (1933), no evidence has been found for a transient rise in respiratory intensity to a temporary maximum at about one and one-half hours after fertilization, as might be expected from the work of Boyd (1928). Moreover, the continuous rise in the rate of oxygen consumption per egg throughout early development is in disagreement with the conclusion of Hyman (1921) that at mid-gastrulation the rate of oxygen consumption per unit of protoplasm is at its highest level for all development. Further increases in rate per egg do occur after mid-gastrulation and these increases are part of a trend in a positive direction which is in evidence before, during, and after the mid-gastrulation period.

It is clear that the rate of increase in respiratory intensity per egg is not constant but varies at different times during development. The first phase of the increase may be correlated with the rapid division of the original one-celled protoplasmic cap into the advanced blastula of many cells. After commencement of gastrulation subsequent increases may primarily result from the addition of new cells to the active embryonic mass by assimilation of yolk material.

In a few cases cell counts have been made of *Fundulus* embryos during early developmental stages (Richards and Porter, 1935; Jones, 1939). In an embryo corresponding to a late high blastula (Stage 9 or about 11 hours after fertilization) there were found about 1,300 cells. Another embryo, an expanding blastula with evidence of a germ ring (Stage 11 or about 18 to 20 hours after fertilization), had about 27,000 cells. For the next 10 to 15 hours the increase in cell number is surprisingly small. At the time of blastopore-closure (Stage 15; 30–35 hours) the total count is at most 35,000 cells. Subsequently the cell number rose relatively rapidly and at the end of the second and third day was about 60,000 and 150,000 respectively.

These data indicate that prior to gastrulation there is a rapid increase in cell number. During gastrulation the total number of cells increases very little. After gastrulation the increase is again quite rapid. There seems to be an obvious correlation between the increase in cell number and the rapid rise in rate of oxygen consumption prior to gastrulation, the falling off during gastrulation, and the rapid rise again after gastrulation.

The results of Trifonova (1937) suggest fundamental differences in the development of perch and of *Fundulus* eggs as far as respiratory rates are concerned. Perch eggs appear to undergo sharp increases in oxygen consumption followed by equally marked decreases at the commencement of gastrulation. Similar fluctuations occur during the time of first appearance of the embryo. Trifonova has concluded that the observed high respiratory rates are directly correlated with fundamental physiological changes occurring during periods of differentiation. On the basis of the present experiments this is not true for *Fundulus*. It is interesting for comparison to note that the results of Brachet (1934), Atlas (1938), and Steffanelli (1938) with amphibian eggs are in agreement with *Fundulus* in showing a continuous rise in respiratory rate per egg without sudden fluctuations during early development.

The demonstration of a constant cyanide-insensitive respiration in the *Fundulus* egg is of some interest. The relative constancy of its absolute value during the first four days of development would tend to indicate that the respiratory increases in early development are primarily the result of increases in the cyanide-sensitive enzyme system. Similar changes in the absolute value of cyanide-sensitive respiration have been found in various developing eggs. The increased respiration caused by fertilization in the sea-urchin eggs is known to be cyanide-sensitive (Runnström, 1930, 1935; Örström, 1932; Korr, 1937). Likewise the respiration of grasshopper eggs in the diapause state is cyanide-insensitive while the difference in respiratory rate between diapause and developing eggs is the result of the relative absence or presence of a cyanide-sensitive oxidation system (Bodine and Boell, 1934).

Since the work of Fisher and others mentioned above has demonstrated the presence of cytochrome-oxidase in the pace-maker mechanism of the embryonic *Fundulus* heart, it is not too great an assumption for the present to state that the probable effect of cyanide on the respiration of *Fundulus* eggs is mediated through the reversible combination of HCN with cytochrome-oxidase. Such combination, by inhibiting the reduction of ferri-cytochrome-oxidase, reduces the oxidations within the cells of the egg to the level of the low cyanide-resistant respiration. Furthermore, the development of *Fundulus* eggs treated with cyanide as described in the results of this paper is similar to the development of eggs subjected to anaerobic conditions as demonstrated in the experiments of Loeb. This is also true in solutions of NaN_3 which inhibit cytochrome-oxidase activity in a manner similar to NaCN. From the close agreement between the work of Loeb and the work presented in this paper, it seems that the effect of NaCN and NaN_3 on development of *Fundulus* is produced by curtailment of cellular oxidations as is true for the effects of anaerobiosis.

Although no attempt was made to ascertain the effect of NaCN or NaN_3 on the respiration of the pelagic eggs, it seems valid to assume that the poisons act by inhibiting cellular oxidations in these eggs. This assumption is again substantiated by the similarity of the effect of NaCN and NaN_3 in suitable concentrations and the effect of oxygen lack as shown in Loeb's experiments with cunner eggs.

The question now arises as to the significance of the cyanide-resistant respiration in development. It is not likely that the development of the *Fundulus* egg in the cyanide or azide solutions can be attributed to the presence of a cyanide- or azide-stable respiration for in Loeb's experiments no oxygen was available to the eggs. In spite of this fact the eggs were able to develop under conditions where a cyanide-stable respiration was of no significance. Furthermore, available comparisons of development of *Fundulus* under the different conditions of the two types of experiment reveal that eggs placed in M/1,000 NaCN shortly after fertilization developed no further than did similar eggs under anaerobic conditions. Obviously there is no evidence to show that the cyanide-stable respiration, which is a considerable proportion of the total respiration in the first day of development (20 to 30 per cent), plays any rôle in the developmental processes of the *Fundulus* egg. No positive, quantitative statements can be made with regard to the possible rôle of the cyanide-stable respiration until parallel experiments using oxygen-lack and cyanide are compared simultaneously on similar batches of eggs. For the present, however, it would appear that in the *Fundulus* egg the particular aerobic processes upon which development ultimately must depend involve the cyanide-sensitive respiratory system. It would not seem apparent that any non-specific respiratory mechanism can provide the necessary energy source for the development of the embryo.

An interesting problem which grows out of these investigations is the mechanism of anaerobic development. Brachet (1934) has suggested, with some supporting evidence, that an oxidative reserve is present in the developing amphibian eggs. This reserve would be some sort of hydrogen-acceptor which could carry out oxidations within the developing egg during anaerobiosis. Whatever the nature of the anaerobic source of energy necessary for development in the *Fundulus* egg, it is clear that under conditions of different rates of oxidation, presumed to be the result of using different concentrations of inhibitors, the energy source is depleted at correspondingly different times. Thus in more dilute poison, eggs presumably respire at greater rates and as a result reach later stages before complete inhibition of development results or else continue development with decreased velocities.

From such results it is conceivable that development depends directly on anaerobic reactions involving the presence of some substance whose synthesis is primarily aerobic. Furthermore, this synthesis seems specifically performed by the cyanide-sensitive respiratory system as indicated above. Anaerobic development may then be assigned to the presence of an excess amount of this substance. Moreover, lowered developmental rates under conditions of reduced respiration within the egg may be thought of as steady states arising from the continuous depletion of the necessary substance and its continuous but retarded rate of synthesis. Under such circumstances the limiting factor in the developmental rate will be the steady state concentration of the substance.

From this viewpoint there is a possible similarity in the action of the inhibitors of cellular respiration on pelagic eggs and on the *Fundulus* egg. It is apparent that the pelagic eggs cannot withstand large reductions in oxidative intensity without undergoing almost immediate inhibition of development. However, the results have demonstrated that in dilute concentrations of poison, where presumably reductions in respiratory rate are not as severe, slow rates of development are possible before complete inhibition occurs. This might mean that in the pelagic eggs as in the *Fundulus* egg there is also some anaerobic source of energy whose utilization becomes manifest during periods of relative anaerobiosis. Or alternately, in contrast to the *Fundulus* egg, the early development of the pelagic eggs may be more directly dependent on completely aerobic mechanisms.

SUMMARY

1. The respiratory rate of the *Fundulus* egg increases during the first two and one-half days of development. There is no temporary maximum rate with subsequent decline shortly after fertilization, nor is there evidence for any striking fluctuations in rate during the period studied.

2. The increases in cellular oxidation in the early development of *Fundulus* may be attributed to increases in cell number and in the amount of material incorporated in the active embryonic mass. These increases in rates of oxidation are not to be correlated with any particular embryonic stage.

3. The respiration of the developing *Fundulus* egg is cyanide-sensitive. The absolute value of the insensitive portion of the respiration remains relatively constant through four days after fertilization. Therefore, the respiratory increases occurring during development are almost entirely in the cyanide-sensitive respiratory system.

4. The earlier observations of Loeb on the relative sensitivity of the *Fundulus* and cunner eggs to anaerobic conditions are confirmed by the use of the respiratory poisons, NaCN and NaN_3. In their sensitivity to these poisons scup and mackerel eggs resemble those of the cunner.

5. *Fundulus* eggs before the end of gastrulation are capable of extensive development in high concentrations of NaCN and NaN_3 which completely and almost immediately inhibit the pelagic eggs. In lower concentrations the pelagic eggs can develop at decreased rates.

6. No differences in sensitivity to cyanide or azide have been found among the eggs at various embryonic stages studied in this paper.

The author is indebted to Dr. David R. Goddard for his stimulating help and advice during the course of this work. Professor B. H. Willier has contributed many helpful suggestions. The U. S. Bureau of Fisheries at Woods Hole kindly provided a liberal supply of mackerel and scup eggs.

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THE PITUITARY GLAND OF THE AFRICAN LUNGFISH, PROTOPTERUS AETHIOPICUS

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Information on the histology of the pituitary gland of the Dipnoi is fragmentary. The early development of the gland in *Lepidosiren* was described by J. G. Kerr (1902) and a sagittal section of the adult organ was included by de Beer (1926) in his monograph on the comparative morphology of the pituitary. Later T. Kerr (1933) gave a more complete account of the embryology of this organ in *Lepidosiren* and included a brief description of the histology of the adult gland. This material had been preserved in formalin for a long time and consequently was not favorable for detailed histological analysis.

Griffiths (1938) has added some additional notes on the development of the pituitary in *Lepidosiren.* His attention, however, was devoted chiefly to the Australian lungfish, *Epiceratodus* (*Neoceratodus*) *forsteri,* and he gave a short account of the development and histology of the gland in this form. Except for a brief reference by J. G. Kerr (1902) to an embryonic stage, the pituitary of *Protopterus* seems to have escaped histological study.

In *Epiceratodus, Lepidosiren* and *Protopterus* the epithelial portion of the gland develops as a solid wedge of epithelial cells derived from the stomodaeal epithelium. This ingrowth extends caudally and comes to lie beneath the brain in a region posterior to the future optic chiasma. Later, a cavity develops within the solid ingrowth and the connection with the superficial ectoderm is lost. The epithelial hypophysis now flattens and elongates to become intimately associated with the posteroventral surface of the infundibulum. Apparently lateral lobes are not developed and the pars tuberalis accordingly is absent in the adult organ.

In *Epiceratodus,* only the anterior end of the epithelial hypophysis is closely appressed to the infundibulum. In *Lepidosiren,* and probably in *Protopterus* also, the entire dorsal surface of the elongated epithelial rudiment is applied to the infundibulum. In *Epiceratodus* the epithelial hypophysis " along the whole of its length, curls around and becomes intimately united to the posterior face of the infundibulum. The cells of the caudal tip become the pars intermedia, whilst growth of the hypophysis, ventral to the hypophysial cavity, results in the large pars anterior" (Griffiths, 1938). However, in the adult the infundibulum is no longer directed backwards but extends vertically from the floor of the brain into a deep sella turcica so that the pars anterior eventually occupies a true anterior position.

In *Lepidosiren* (Kerr, 1933) the portion of the epithelial hypophysis dorsal to the cavity and in contact with the infundibulum remains relatively thin and the boundary between the nervous and epithelial component is soon broken down. Ventral to the cavity the gland is greatly thickened. These two epithelial differentiations foreshadow the pars intermedia and pars anterior respectively. However, de Beer (1926) fails to show a cavity in the adult pituitary of *Lepidosiren* (Fig. 70). There is apparently little change in the relative position of the infundibulum in the adult. It is directed ventro-caudally with the epithelial components lying ventral to the neural lobe. The adult gland is a wedge-shaped organ attached at its broad end to the tip of the infundibulum and sunk into a shallow depression in the cranial floor.

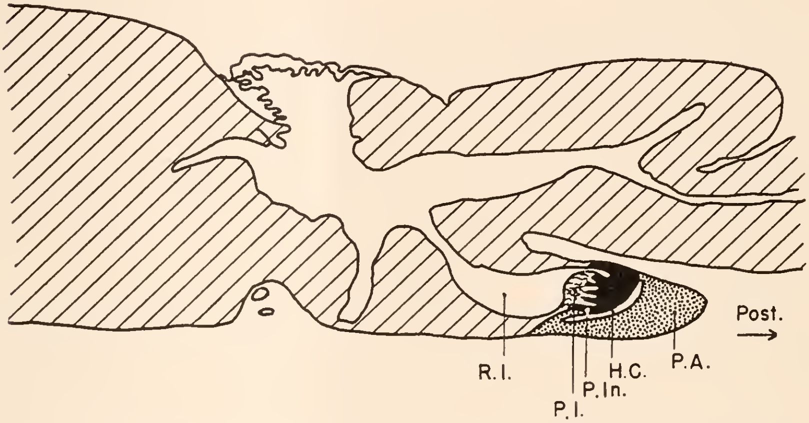

FIG. 1. Diagrammatic sketch of a sagittal section of the brain of *Protopterus* showing the relation of the pituitary gland to the infundibulum. The regional differentiations within the pituitary are also represented: *R.I.*, recessus infundibularis; *P.I.*, processus infundibularis; *P.In.*, pars intermedia; *P.A.*, pars anterior; *H.C.*, hypophysial cavity. × 9.

Materials

This study of the pituitary of *Protopterus* is based on two male fish (14 inches long) which were obtained from the General Biological Supply House, Inc. and kept in the laboratory for several months. One died at night and the pituitary was dissected out and fixed the following morning. The other was killed and the tissue fixed immediately. In both cases formol-sublimate was used. The glands were left attached to the floor of the brain and were sectioned sagittally. All tissue was stained by the azan method of Heidenhain. The fixation was excellent in the freshly fixed gland but some cytolysis had occurred in the other which was not obtained until several hours after death.

Description

In a ventral view of the brain only the infundibular process and the flattened, ovoid pars anterior of the pituitary are visible (Fig. 2). The gland lies close to the brain and the floor of the cranium is not depressed in this region to form a sella. The pituitary is located well posterior to the optic chiasma and is directed caudally. Its topographical relation to the brain is clearly seen in sagittal sections (Fig. 1). The infundibular process is wide and flattened, well delimited from the floor of the brain and projects caudally. The portion of the process associated with the epithelial component of the gland is thin-walled except in a medial postero-ventral region applied to the pars intermedia, where a considerable thickening occurs. The thickening is due to a local massing of fibrous tissue apparently similar to that of the posterior lobe of other vertebrates (Fig. 3). Irregular slender outgrowths from the saccular infundibular recess invade the compact tissue. They appear to be homologous with the so-called sinuses in *Lepidosiren* (Kerr, 1933) and are readily distinguished from empty blood vessels by the ependymal lining.

The epithelial portion of the pituitary possesses a conspicuous hypophysial cavity which incompletely separates an antero-dorsal intermediate zone from a flattened, elongated ventro-caudal pars distalis. The cavity does not extend completely to the periphery of the gland and in this region the tissues of the pars intermedia and pars distalis are not sharply segregated from one another.

The pars intermedia is composed of closely apposed epithelial diverticula or cords which appear to be related to the hypophysial cavity. Their appearance suggests that the relatively thick intermedia may have been formed by epithelial outgrowths from the adjoining wall of the cavity. Centrally, the cords or diverticula are interdigitated with the compact fibrous portion of the infundibular process. More peripherally they terminate close to the ependymal lining of the recess. No blood vessels were seen in the pars intermedia but in many instances the pars intermedia tissue is not far distant from the vessels of the infundibular process.

The cells of the intermedia are usually elongated and compactly arranged at right angles to the long axis of the cords, or to the surface of the lumina of the diverticula. The majority of them are lightly basophilic, i.e., react with anilin blue. Some cells react with orange G. These are rounded and tend to be concentrated at the tips of the cords which are embedded in the infundibular process but they may also be scattered along the surfaces of the cords that are separated by fibrous extensions from the process (Fig. 4).

The pars anterior is composed of irregular cords of cells invested by a delicate reticular network. A rich plexus of blood vessels is present. Three types of chromophile cells may be recognized. The basophiles are deeply stained by anilin blue. The acidophiles may be divided tinctorially into two groups. Some react specifically with azocarmine, others with orange G. The granulation in the cells reacting with azocarmine appears slightly coarser than that of the orange-stained cells. The significance of the differential staining of the acidophiles is not known. Chromophobes are relatively scarce.

The different classes of cells are not uniformly distributed throughout the pars anterior. The basophiles tend to be concentrated in a

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Explanation of Figures

Fig. 2. Ventral view of the brain showing the infundibular process (*P.I.*) and caudal flattened anterior lobe (*P.A.*). × 6.

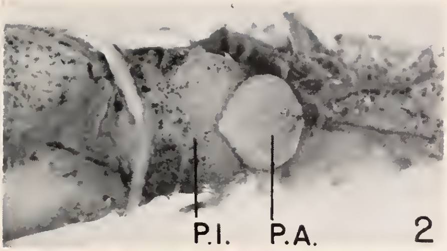
Fig. 3. Area of the thickened wall of the infundibular process showing the fibrous tissue and ependymal lining of the infundibular recess. × 256.

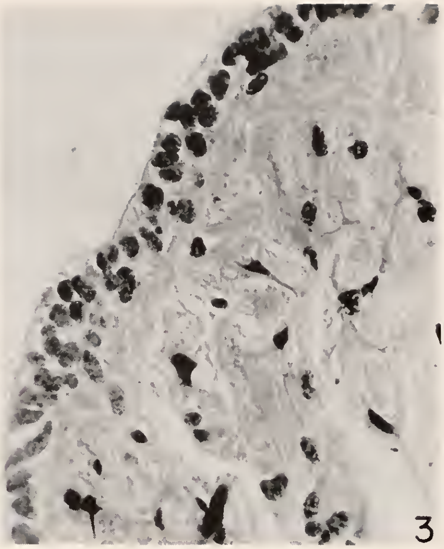
Fig. 4. Tangential section of an epithelial cord of the pars intermedia showing the superficial, rounded, acidophilic cells (*O*) and the deeper elongated basophilic cells cut transversely (*B*). × 768.

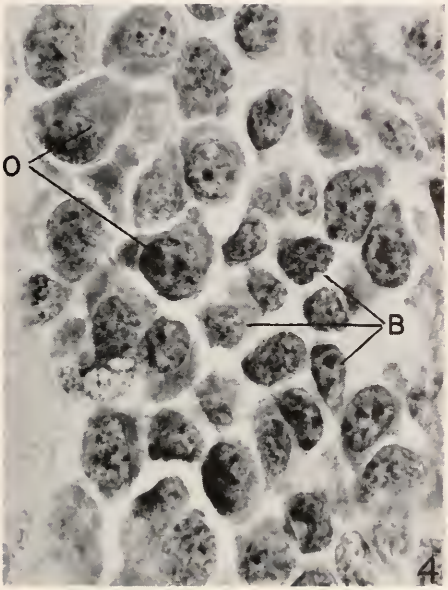
Fig. 5. A group of the elongated acidophiles from a lateral wing of the pars anterior: cells stained with azocarmine appear black (*C*); those stained with orange G are gray (*O*). × 768.

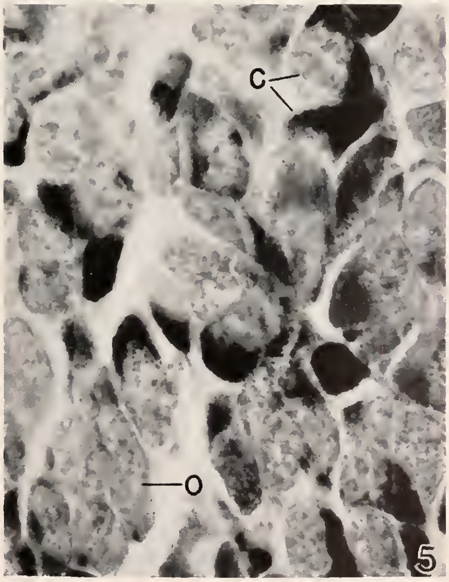
Fig. 6. An area from the median dorsal region of the pars anterior showing the predominance of basophiles (*B*) with occasional acidophiles stained with orange G (*O*). × 768.

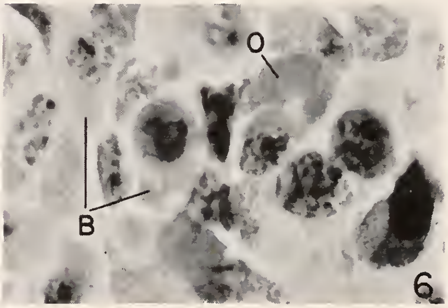
Fig. 7. Area from the median, ventral region of the pars anterior showing a group of elongated basophiles and an occasional acidophile (*O*). Erythrocytes in the capillaries are deep black. × 768.











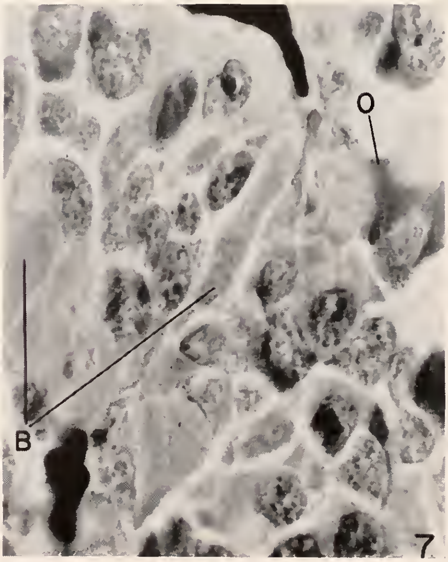


PLATE I

superficial medial band which extends along both the dorsal and ventral surfaces of the pars anterior (Figs. 6, 7). Centrally acidophiles staining with orange G predominate while laterally in the more flattened winglike extensions the carmine-staining cells are numerous (Fig. 5).

A careful study of complete serial sections of these two specimens failed to reveal any evidence of the presence of a typical pars tuberalis, either attached to the pituitary gland or isolated in the adjoining tissue of the brain. The extreme anterior tip of the pars distalis, as seen in medial sagittal sections, is composed chiefly of lightly basophilic cells with occasional orange G-acidophiles. The location is slightly suggestive of position of the pars tuberalis in urodeles and non-metamorphosed anura but the region is single and median rather than paired. It may, however, only represent a marginal region of incomplete differentiation lacking characteristic basophiles and acidophiles. There is no true saccus vasculosus.

Discussion

The presence of a lumen in the pituitary glands of all three Dipnoi is a matter of interest. In each case the epithelial component develops as a solid ectodermal ingrowth and secondarily acquires a lumen which persists. Furthermore, in the final differentiation of the gland the lumen tends to occupy the position typical of many vertebrates between the pars intermedia and the anterior lobe proper. In *Epiceratodus*, however, some anterior lobe tissue is found on the neural side of the lumen in association with the pars intermedia, but in *Lepidosiren* and *Protopterus* only intermediate tissue is found in this location. The phylogenetic significance of the lumen, in relation to its mode of development, either as a remnant of Rathke's pouch or as a secondary schizo-cavity, has been discussed by de Beer (1926) and Griffiths (1938), but these authors failed to find any adequate explanation of the two modes of origin.

In its general histological appearance, its caudal position and the topographical relations of infundibular process, pars intermedia and pars anterior, the pituitary gland of the Dipnoi closely resembles that of Amphibia. In the Amphibia there is also a solid ectodermal ingrowth, but a lumen is not secondarily developed. Nevertheless, the segregation of intermediate and anterior lobe tissue occurs almost as regularly as when a lumen is present. However, in birds a Rathke's pouch is formed but the lumen disappears early and no definitive pars intermediate is present in the adult gland. The further complications observed in teleosts, elasmobranchs, cyclostomes, *Polypterus* and certain mammals (manatee, whale and armadillo lack a pars intermedia) throw no light on this general problem but rather tend to increase the confusion.

Some reference should also be made to the position of the pituitary gland in relation to the infundibulum. In *Epiceratodus* the infundibular process extends vertically from the floor of the brain. The anterior lobe is truly anterior to it and the entire gland occupies a deep sella. In *Lepidosiren* the entire gland lies caudal to the infundibular recess with the pars intermedia and pars distalis lying ventral to the thickened dorso-caudal wall of the infundibular process. The gland occupies a shallow sella. In *Protopterus* the infundibular process is directed caudally and the epithelial portion of the pituitary lies almost directly posterior to the process. The entire gland is close to the brain and there is no depression in the floor of the cranium. The developmental factors influencing the orientation of the pituitary gland with reference to the infundibulum are not known.

Summary

This study is based on two male specimens, 14 inches long. The flattened pituitary gland lies close to the brain and is located caudally to the infundibulum. There is no sella turcica in the cranial floor.

In a ventral view of the brain only the infundibular process and the flattened ovoid pars anterior are visible. The infundibular process is wide and thin-walled except in a median ventro-caudal region applied to the pars intermedia, where a considerable thickening occurs. The epithelial portion of the gland possesses a conspicuous hypophysial cavity which almost completely separates an antero-dorsal intermediate zone from a more elongated ventro-caudal pars distalis. The pars intermedia is composed of open or closed epithelial diverticula which are associated with the hypophysial cavity and are interdigitated with the compact fibrous portion of the infundibular process. The pars distalis is composed of cords of epithelial cells.

The majority of the intermedia cells are basophilic but a number of cells reacting with orange G are present, especially in the region immediately in contact with the neural tissue. In the pars distalis chromophobes are rarely seen. After Heidenhain's azan method three types of chromophilic cells may be distinguished, deeply staining basophiles, acidophiles selectively stained with orange G and acidophiles reacting with azocarmine.

There is no pars tuberalis or saccus vasculosus.

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A CILIATE PROTOZOON PARASITIC IN THE CENTRAL NERVOUS SYSTEM OF LARVAL AMBYSTOMA[1]

WALDO SHUMWAY

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Some years ago a graduate student, Mr. C. W. Clancy, while studying serial sections of *Ambystoma texanum* (Matthes) for an embryological thesis, called my attention to some curious bodies in the ventricles of the brain. They proved to be large ciliates, and since the occurrence of ciliate protozoa in the cerebrospinal fluid is almost unique, I have been on the outlook for another opportunity to observe them. Several years having elapsed without further observations, it seems desirable to put the case on record for the benefit of other investigators.

The *Ambystoma* larva was raised in the laboratory from eggs collected locally by Dr. J. M. Sanders and prepared by him in connection with his thesis on the development of the amphibian thyroid gland. The larva measured 12 mm. before fixation and corresponds to Harrison's stage 40. It was fixed in Smith's formol-bichrome-acetic (frog) mixture, and frontal paraffin sections 10 μ thick were stained with my modification of Cajal's basic fuchsin and picro-indigo-carmine. One slide of the series has since had the stain removed and replaced by Heidenhain's haematoxylin.

The infection is very heavy (Plate I, 1 and 2). Sections of 42 different individuals were counted in one section of the larva selected at random from the 95 serial sections containing portions of the central nervous system. In this particular section 28 ciliates were in the cerebrospinal fluid, 9 in the tissues of the brain and cord, and 5 in the meningeal fluid. No parasites were discovered in the epiphysis, eyeballs or cerebrospinal ganglia, nor in any other part of the body.

A search for similar parasites in 65 sets of serial sections of *Ambystoma* larvae between 5 and 20 mm. in length collected from the same locality has proven fruitless.

While recognizing the difficulty of making a satisfactory identification of species from prepared material only, the excellent preservation of the material leads me to assign the parasites to the genus *Glaucoma*. The general shape of the body is flask-like (Text Fig. 1). The anterior end is sub-cylindrical and tapers gradually to a rounded extremity. Transverse sections show approximately 20 longitudinal rows of cilia located in longitudinal grooves and with large basal bodies embedded in the ectoplasm.

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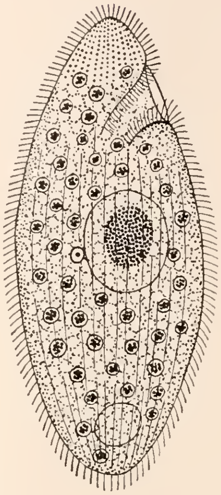
[1] Contributions from the Zoölogical Laboratories, University of Illinois, no. 541.



Text Fig. 1. *Glaucoma sp.* (?), composite drawing from several sections. × 1,000.

The cytostome is located about one-quarter of the body length from the anterior end. It is usually open in sections and leads to a large cytopharynx which is sometimes everted. The cytostome is surrounded by larger cilia and appears to contain two undulating membranes. It is capable of great extension and in one specimen a brain cell is being ingested (Plate I, 4).

The body of the ciliate is much thicker posterior to the cytostome and is ciliated on both dorsal and ventral surfaces. It is filled with numerous food vacuoles containing pigment granules identical with those contained in the tissues of the host. In some individuals a clear space in the

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Explanation of Plate I

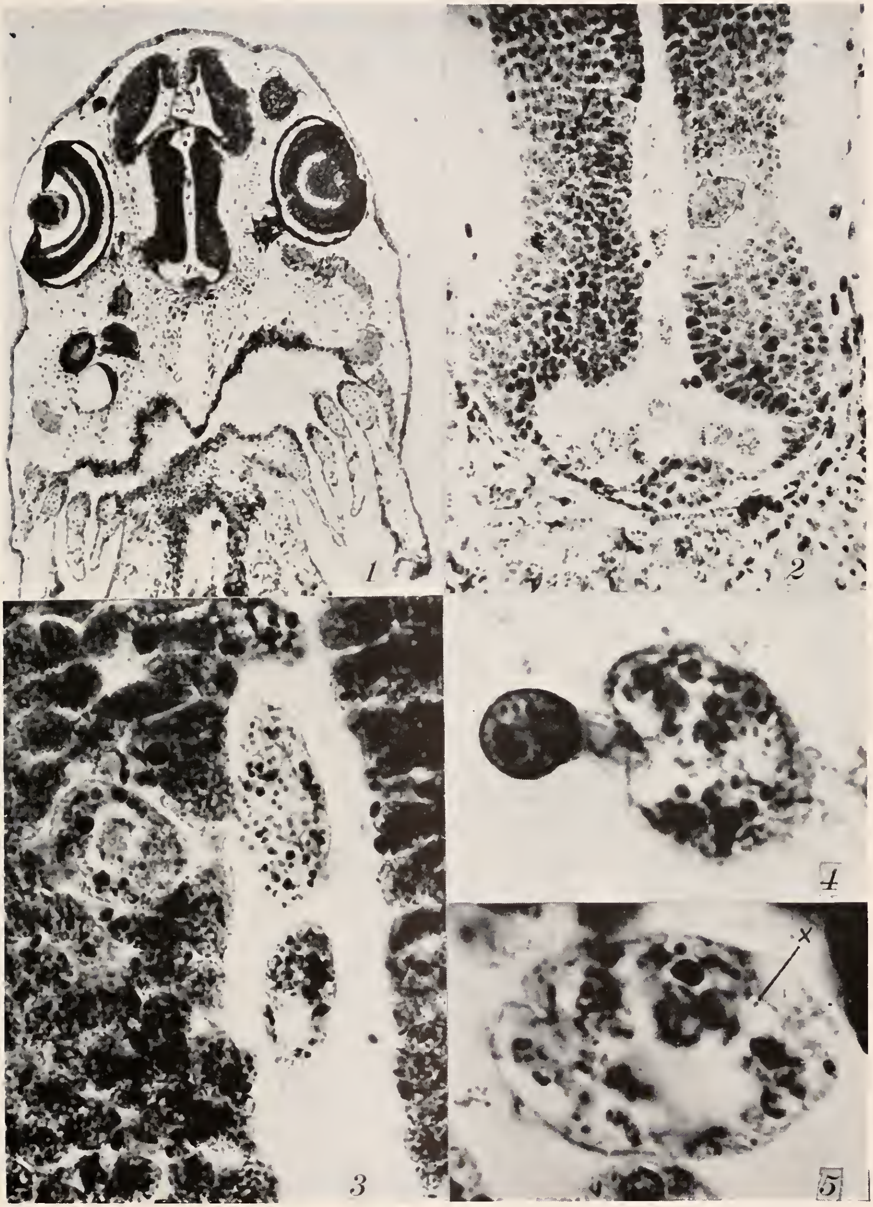
Fig. 1. Frontal section of 12-mm. larva of *Ambystoma texanum* showing site of parasitism. × 37.

Fig. 2. Detail of same showing abundance of *Glaucoma.* × 148.

Fig. 3. Detail of spinal cord showing two free-swimming specimens and one in wall of spinal cord. × 518.

Fig. 4. Section through free-swimming individual ingesting brain cell. × 1046.

Fig. 5. Section through free-swimming individual to show nuclear apparatus. Micronucleus at X. × 1382.



Photographs by George Svihla

PLATE I

posterior end of the body suggests the presence in life of a large contractile vacuole. The posterior end of the body is rounded.

A single macronucleus, central and spherical, lies in a clear area. It is filled with small granules and resembles in all respects the figures of *Glaucoma* macronuclei encountered in the literature. There is a single micronucleus, excessively minute, a small granule in a clear area, quite characteristic of *Glaucoma* (Plate I, 5).

One individual appears to be in an early stage of fission. The macronucleus is elongate and a transverse constriction is apparent. The micronuclei are connected by a delicate strand. No conjugating individuals were found.

Many of the individuals found in the tissue of the brain and cord had rounded up (Plate I, 3) but nothing in the nature of a cyst wall could be demonstrated.

Average dimensions, based on twenty measured individuals are: length 60 μ, breadth 25 μ, depth 17 μ. Rounded individuals in the brain tissue averaged 32 μ in diameter.

There can be no doubt that the organisms have adopted a parasitic mode of life. Otherwise I can see no explanation for the universal appearance of host pigment granules in the food vacuoles. In several cases individuals are clustered around detached bits of host tissue in the ventricles of the brain, and in one case the ciliate is actually ingesting a host cell. But beyond the presence of a few leucocytes in the cerebrospinal fluid there are no indications of reactions by the host. Certainly there are no lesions of the brain or cord, which appear perfectly normal in all respects.

The amazing ability of the protozoön to penetrate the walls of the brain is indicated by its presence everywhere from the ependymal layer to the meningeal space. In one case the parasite has just entered the brain wall and the latter has not yet closed together behind it.

The question as to how the parasite gained entrance to this curious new habitat is extremely puzzling. At first one would be inclined to think that one or more individuals were trapped by the closing of the neural folds. But in *Ambystoma* this event takes place while the embryo is still protected by its egg jelly. Possibly the infection takes place after hatching and the parasites make their way from skin or digestive tract through the tissues into the central nervous system. On this point I have, however, no direct evidence.

Turning to other notices of parasitism by *Glaucoma,* the one presenting the greatest similarity to the one here reported is by Epstein (1926). He reports the discovery of a *Glaucoma*-like infusorian parasitic in the spinal cord, brain, and eyeballs of young larvae of the bream, *Abramis brama* Linn. His own studies were made on prepared sections of the host (sublimate-acetic fixation). The parasites were smaller ($35 \times 20\,\mu$) and he was unable to identify a micronucleus. He compares the inclusions of the food vacuoles to yolk granules. His figure illustrating the posterior end of the parasite is almost exactly similar to those which I identify on the basis of serial sections as the anterior end.

Epstein cites field notes (of Dr. Kryjanowsky) to account for the manner of infection. According to these, the parasites are found first in the yolk sac from which they make their way to the gut, the primordium of the liver, the hepatic circulation, into the heart, and thence, via the capillaries of the central nervous system, to the brain and cord. They are found in 2 or 3 per cent of the larvae during the first week of larval life, and were recognized by a milky color of the head and inability to live in the aquaria. The parasites destroy the spinal cord and surrounding tissues within two or three days of establishing themselves in the central nervous system.

While Epstein's case is the only other record of a vertebrate larva parasitized by *Glaucoma,* there are several instances of invertebrate hosts. MacArthur (1922) reports *G. pyriformis* as a parasite in the body cavity of larval mosquitos (*Theobaldia annulata*): Treillard and Lwoff (1924) the same species as a parasite in larvae of *Chironomus plumosus.* Penard (1922) identifies as a new species (*G. parasiticum*) a parasite in the body cavity of *Gammarus pulex.* Wenyon (1926), in a comprehensive review of parasitism by *Glaucoma,* suggests that to this genus may be referred also the parasites *Lambornella stegomyiae,* Keilin 1921, discovered by Lamborn (1921) in *Aedes scutellaris,* and *Ophryoglena collini,* Lichtenstein 1921, a parasite of *Boetis.*

It is generally recognized that *Glaucoma* is quite tolerant of CO_2. Kofoid (1908) notes its presence in the Illinois river as a member of the plankton during the time of bacterial increase. Lwoff (1924) was able to inoculate successfully caterpillars of *Galleria mellonella* with *Glaucoma pyriformis.* Wenyon (1926), on the other hand, cultured in pond water parasites taken from *Theobaldia annulata.* Janda and Jírovek have recently (1937) reported success in inoculating 14 species of insects with a pure culture of *Glaucoma pyriformis* but failure when the same experiment was attempted with fish and amphibia.

This note records further evidence that the free-living ciliate *Glaucoma* is able to exist as a facultative parasite not only in the coelomic fluid of arthropods but also in the cerebrospinal fluid of larval fish and amphibia.

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THE LUNGS OF THE LARGER CETACEA COMPARED TO THOSE OF SMALLER SPECIES[1]

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INTRODUCTION

In previous publications we have reported on the structure of the lung in *Tursiops truncatus* (Wislocki, 1929) and in *Delphinapterus leucas* (Bonin and Bélanger, 1939). The former belongs to the family of the Delphinidae, whereas the latter is a member of the Delphinapteridae. Both are small species of Cetacea belonging to the sub-order of Odontoceti or toothed whales.

In each case the lungs were quite comparable, the following being their important features:

1. The lungs show no distinct lobulation, although dense collagenous tissue is very abundant, surrounding the air sacs, air ducts, and blood vessels.

2. The presence of an extensive cartilaginous armature whose elements are fused together and encircle the bronchi and bronchioles down to their terminations where they open into the respiratory sacs.

3. The presence of a system of myo-elastic sphincters disposed at short intervals in the smallest bronchioles, the last one of them marking the entrance to the alveolar complex.

4. The presence of a double capillary bed, one on each side of the thick and mostly collagenous interalveolar septum.

Through the interest and generosity of Dr. E. M. K. Geiling, we have now been able to study the lungs of three species of large whales: the blue whale (*Balaenoptera sibaldii*), the finback whale (*Balaenoptera physalus*), and the sperm whale (*Physeter megalocephalus*). The lungs of these larger Cetacea differ markedly in several important respects from those of the smaller species previously examined, and it is the purpose of the present note to set forth briefly the results of our comparative study.

MATERIAL

The material consists of slabs of lung tissue, fixed in 10 per cent formalin, from the blue whale (*Balaenoptera sibaldii*), the finback whale

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[1] Partially aided by the Fondation Rougier-Armandie.

[2] Fellow of the Government of Quebec (Canada).

(*Balaenoptera physalus*), and the sperm whale (*Physeter megalocephalus*). The specimens were obtained by Dr. E. M. K. Geiling at a whaling station in British Columbia.

The fixation of the material is relatively poor, due to the fact that eight to twenty-four hours elapsed before autopsy. Nevertheless, the specimens from the lungs of a blue whale are quite adequately preserved, and they constitute the main basis for the present report.

Large pieces of tissue have been imbedded in celloidin, cut from 10 to 20 micra, and stained with hematoxylin-eosin, azur, resorcin-fuchsin and hematoxylin-ponceau-acid fuchsin-anilin blue (Masson).

Description

The lungs of the three species studied were found to be quite similar, and the following description applies to all of them.

Air Ducts

The bronchi and bronchioles show the following structure:

1. In the bronchi and larger bronchioles a stratified or pseudostratified epithelial lining.

2. A tunica propria composed of loose collagenous connective tissue and containing a great number of small blood vessels and occasional mucous glands.

Plate I

Fig. 1. Lung of the blue whale (*Belaenoptera sibaldii*) showing a bronchiole which is surrounded by cartilage and which terminates in an elaborate respiratory complex. Observe the relatively large size of the air sacs and the thickness of the septa bounding them. Notice that there are no myo-elastic sphincters in the terminal bronchiole. Masson stain. × 13.

Fig. 2. Lung of the white whale (*Delphinapterus leucas*) for comparison with that of the blue whale (Fig. 1). The terminal bronchioles of the white whale contain typical myo-elastic sphincters, several of which are visible in the photograph, whereas the bronchioles of the blue whale contain none. In both species the cartilaginous armature is extensive and extends to the terminations of the bronchioles. Masson stain. × 35.

Fig. 3. A typical myo-elastic bundle (*m.b.*) in one of the septa protruding into an air sac of the blue whale. Typical elongated nuclei are visible in the muscle fibers. Compare this figure with Fig. 6 in which a number of similar muscle bundles are visible at lower magnification. Masson stain. × 167.

Fig. 4. A septum bounding an air sac in the small white whale for comparison with a septum in the blue whale (Fig. 3). The two photographs are at the same magnification. Observe that myo-elastic tissue (*m.b.*) is present in the septum of the white whale, but that it is exceedingly delicate when compared with the heavy myo-elastic bundles occurring in the blue whale. Masson stain. × 167.

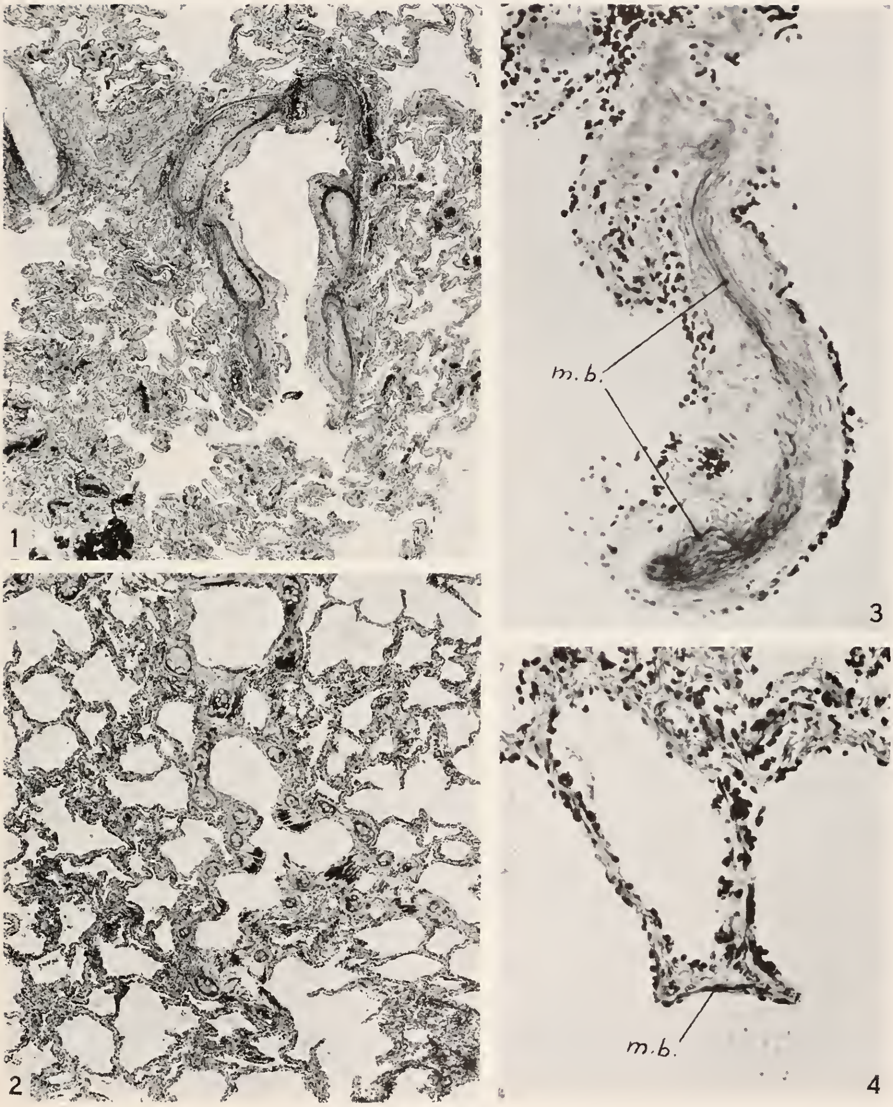

PLATE I

3. A stout band of circular smooth muscle in the tunica propria. This layer increases in relative amount as one approaches the terminal bronchioles.

4. A strong cartilaginous armature consisting of large pieces of apparently hyaline cartilage, which are linked together, and are surrounded by a heavy sheath of elastic fibers. The cartilage extends down to the openings of the bronchioles into the air sacs (Fig. 1).

Respiratory Complex

The air sacs, compared with terrestrial mammals, are enormous and their walls tremendously thick (Figs. 5 and 7). On examination it is possible to distinguish: (1) a large space which forms the central undivided portion of the respiratory complex, and (2) small spaces which are numerous and lead off from the larger space, forming a series of pockets which are undoubtedly alveoli (Figs. 5 and 7). The following table presents the mean diameters of a series of these larger and smaller spaces which together constitute the air sacs. We refrain advisedly at this time from homologizing the large undivided portion of the air sac with the segments designated in other mammals as either "ductus alveolaris" or "atrium." Future reconstructions of the respiratory sacs, which we plan to undertake, should clarify these questions of homologies with terrestrial mammals.

	Large spaces	Alveolar spaces
Blue Whale	4.71 mm.	0.40
Finback Whale	2.00	0.45
White Whale	0.90	0.35

These observations indicate that the diameters of the large spaces vary considerably in the several species. The data suggest, moreover,

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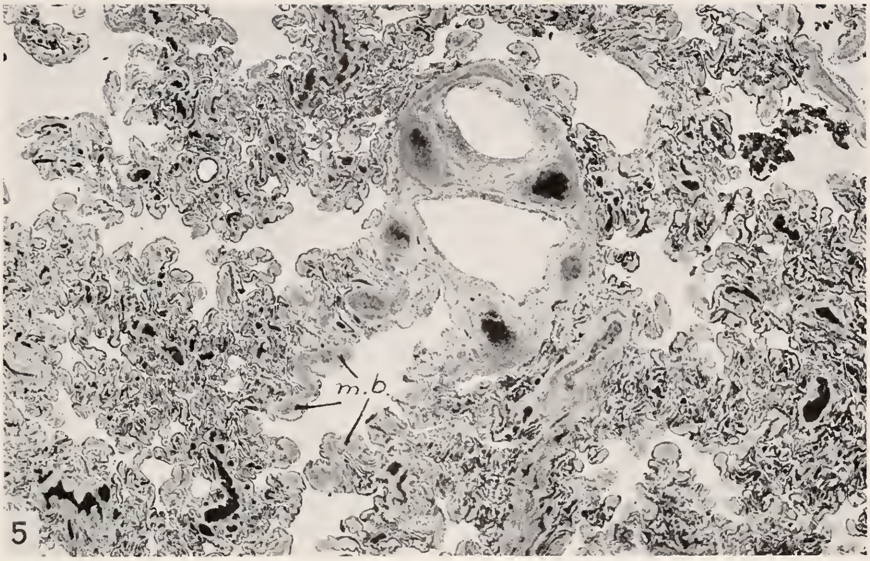
PLATE II

FIG. 5. Lung of blue whale to show the cartilaginous armature around the bronchioles, the tremendous size of the air sac and the exceedingly thick septa bounding the air sacs. The larger septa protruding into the air sac contain myo-elastic bundles (*m.b.*). Hematoxylin and eosin. × 16.

FIG. 6. A portion of the wall between two air sacs in the blue whale, showing a number of myo-elastic bundles (*m.b.*) in the larger septa which protrude into the air sac. In the septa separating the air sacs, note the double capillary bed with intervening collagenous tissue. Hematoxylin and eosin. × 66.

FIG. 7. Lung of blue whale, stained with Weigert's elastic tissue stain, to demonstrate that the bundles, typically seen in the larger septa which protrude into the air sacs, are composed of elastic tissue, besides smooth muscle, the two components together constituting myo-elastic bundles. Compare Fig. 7 with Figs. 3, 5 and 6 in which the muscular component has been principally brought out by the stains employed. × 16.



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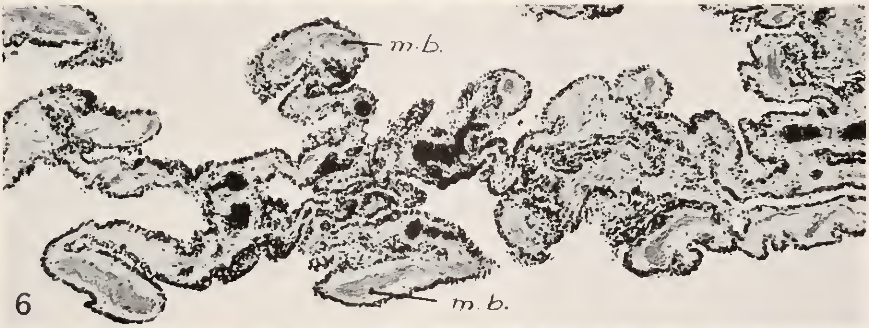

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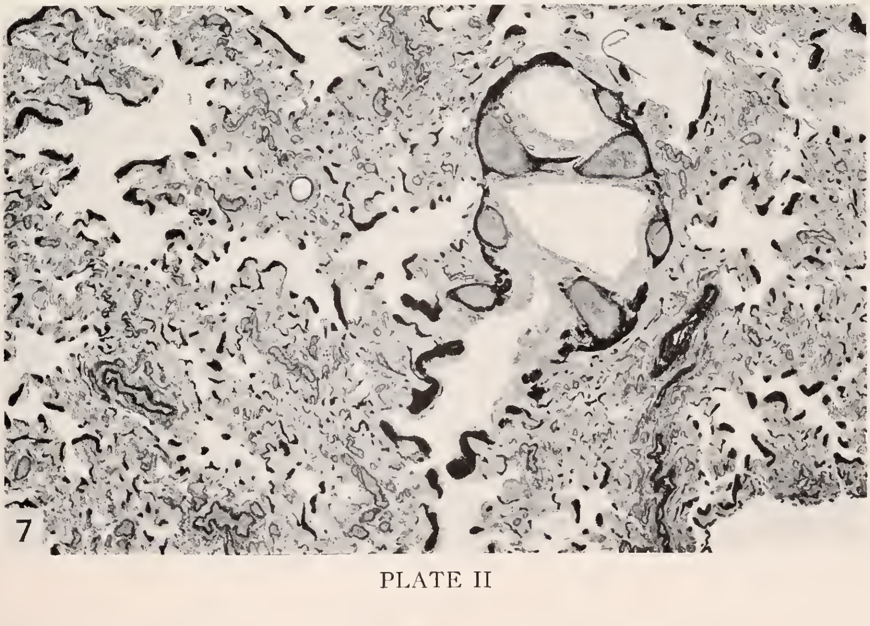

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PLATE II

that the air sacs are more voluminous in larger whales (blue and finback whales) than in the smaller species (white whale). The alveolar spaces, on the contrary, are quite constant in size in the three species.

The interalveolar septa are excessively thick in the Cetacea and their diameters are greater in the larger than in the smaller species (Figs. 1 and 2). Moreover, the septa projecting into that portion of the air sac near the entrance of the bronchiole exceed in thickness the diameter of the interalveolar septa (Fig. 6, *m.b.*). Measurements of the average diameter of the septa in several species are presented in the following table:

	Large septa	Interalveolar septa
Blue Whale	240 μ	60 μ
Finback Whale	60 μ	20 μ
White Whale	30 μ	20 μ
Tursiops	75 μ	20 μ

The interalveolar septa are bounded on both sides by an extensive capillary bed (Fig. 6) over which no epithelial lining can be positively identified in either the blue whale, sperm whale, or finback whale. The poor preservation of the tissue may possibly account for the failure to recognize the epithelium.

The most striking feature of the larger septa, which project into that portion of the respiratory sac adjacent to the bronchiole in the larger whales, is the presence of heavy bundles of tissue which are shown by Masson's trichrome stain to be composed of both muscular and elastic fibers intimately intermingled (Figs. 3, 5, 6 and 7). These myo-elastic bundles present a wavy appearance (Figs. 3 and 6, *m.b.*) and a definitely circular orientation in reference to the long axis of the air sac. We have demonstrated the myo-elastic nature of these bundles in the three species of whales studied. It is noteworthy, on the contrary, that the system of myo-elastic sphincters which is so characteristic of the smallest bronchioles of the small Cetacea (*Tursiops truncatus* and *Delphinapterus leucas*) (Fig. 2) is totally lacking in the three large whales (Fig. 1).

Discussion

The lungs of the Cetacea show certain modifications which are common to all species, more especially the presence of an extensive cartilaginous armature, the possession of large air sacs with thick walls, and the presence of a double capillary net in the interalveolar septa.

We have found important differences, however, in the manner in which the myo-elastic tissue is arranged to produce closure of the respiratory portion of the lung. In the small harbor porpoise (*Phocaena communis*), Lacoste and Baudrimont (1933) have seen no particular myo-elastic closing apparatus within the smaller bronchioles or air sacs. In the bottle-nosed porpoise (*Tursiops;* Wislocki, 1929) and the white whale (*Delphinapterus;* Bonin and Bélanger, 1939), we have described a system of myo-elastic sphincters occupying the smallest bronchioles (Fig. 2). Similar structures had been previously observed in the Mediterranean dolphin by both Fiebiger (1916) and Lacoste and Baudrimont (1926).

In the large whales the only previous references to the histology of the lungs are brief accounts by Laurie (1933) and Haynes and Laurie (1937) on the lungs of the finback, humpback, sperm and Southern right whales. These authors describe merely a " heavy elastic bundle circumscribing each infundibulum." We are able to demonstrate in the finback and blue whales that the bundles mentioned by Haynes and Laurie are not purely elastic, but constitute conspicuous, well-organized myo-elastic structures situated in the large septa which project into the central portion of the air sacs. Consequently we conclude that the Cetacea are provided with two types of closing mechanism: (1) in the larger whales an arrangement of stout myo-elastic bundles present in the septa which surround and project into the proximal portion of the air sacs (Figs. 3, 5, 6 and 7) and (2) in the smaller Cetacea a characteristic system of sphincters located in the smallest bronchioles (Fig. 2). In the first structure, however, the elastic component appears to predominate, whereas in the myo-elastic sphincters characteristic of the smaller species the muscle is more abundant.

It should be noted, moreover, that in those Cetacea which possess myo-elastic sphincters in the bronchioles, traces of myo-elastic bundles in the walls of the air sacs are also present (Fig. 4). In the large whales, however, in which the tremendously hypertrophied myo-elastic bundles exist in the larger septa projecting into the air sacs, no trace whatsoever of myo-elastic sphincters in the bronchioles can be demonstrated. Thus the bundles are a more generalized feature, whereas the sphincters must be regarded as a specialization restricted to relatively few species. Indeed, the bundles may prove to be related to and derived from the delicate strands of muscle, known to exist in terrestrial mammals, in the septa which partially subdivide that segment of the air sacs generally called the alveolar duct. The myo-elastic bronchiolar sphincters are doubtlessly differentiated from the sheet of smooth muscle which occurs in the bronchiolar wall of all mammals.

In conclusion, we wish to point out that the muscular component of the myo-elastic bundles, which we have described in the larger whales, provides in all probability a powerful mechanism for closure of the air sacs. On the other hand, the elastic component contributes, it would seem apparent, to rapid evacuation of the air during expiration. In the case of the myo-elastic sphincters of the smaller whales, it seems clear that the muscular and elastic components subserve similar purposes.

Summary and Conclusions

The following comparisons can now be instituted between the lungs of the larger whales and those of the several smaller species previously studied:

1. The bronchial tree in all Cetacea which have been examined is enclosed throughout by fused links of cartilage which render it relatively rigid.

2. The air sacs are excessively large but variable in size in different species. The alveolar component of the air sacs, on the contrary, appears to be constant in size in the several species examined.

3. Each air sac has its own capillary net, the interalveolar septum being lined by capillaries on each side.

4. In the large whales (*Balaenoptera sibaldii, Balaenoptera physalus, Physeter megalocephalus*) the septa projecting into the central, proximal portion of the air sacs contain heavy myo-elastic bundles. No system of myo-elastic valves is present in the terminal bronchioles.

5. In the smaller Cetacea (*Delphinus, Tursiops* and *Delphinapterus*) similar, but very much more delicate, myo-elastic bundles exist in the septa projecting into the proximal portion of the air sacs. Characteristic, however, is the presence in these species of an elaborate system of myo-elastic valves in the smallest bronchioles.

It may be concluded that the myo-elastic bundles are a more generalized feature than the myo-elastic bronchiolar sphincters. The former, present to some degree in all members of the Cetacea, undergo a tremendous hypertrophy in large whales, whereas the myo-elastic sphincters are a specialized structure found exclusively in some of the smaller species of Cetacea. It seems likely that the myo-elastic bundles will prove to be homologous to the extremely delicate strands of muscle described in the ductus alveolaris of many other mammals.

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BUDS INDUCED BY IMPLANTS OF ANTERIOR NERVE CORD AND NEIGHBORING TISSUES INSERTED AT VARIOUS LEVELS IN CLYMENELLA TORQUATA

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It has been shown (Sayles, 1939) that in *Clymenella torquata* a bud may be produced as the result of the implantation of a piece of nerve cord and some neighboring tissues from an adult, non-regenerating animal of the same species. In all of the cases reported the implants were inserted in the dorso-lateral region of the body wall of the thirteenth segment. Although a number of implants were from the anterior region of the donor, none of the buds in any way resembled a head. The data presented pointed clearly to the fact that, at the thirteenth segment, the host and not the implant determined the nature of the bud to be found.

Similar experiments have now been carried out inserting implants in various regions of the host from the first to the fifteenth segment. In each case the operation was performed in essentially the same manner as described in the earlier report. The present paper covers those operations in which the source of the implant was the anterior region of the donor, levels at which ordinary regeneration occurs only in the anterior direction. This would include segments 1 to 7.

This work was carried on at the Marine Biological Laboratory, Woods Hole. It was made possible through the generous coöperation of the staff of the Supply Department at the Laboratory. Their task of securing large numbers of intact worms of the size range satisfactory for this work was at best a tedious one.

RESULTS

In making this study, the factor of orientation of the implant was taken into consideration. Out of a total of 556 operations, 398 of the implants were inserted so that the posterior end of each was pushed into the coelomic cavity while the original anterior end healed into the integument with its tip exposed at the surface. In 141 other cases it was the posterior end which was at the surface. The remaining 17 cases were early experiments and no records of this factor were made.

In the following discussion it will be necessary to give the exact source of the implant in each case. For the sake of brevity, therefore, the expression "implant 2–4," for example, will be used to mean "implant from segments 2–4 of the donor."

Anterior End of Implant Exposed

The general results of those cases where the implants were inserted with their anterior ends exposed are summarized in Table I. One of the interesting points brought out here is that there is a transition zone in the region of segments 10–12. Anterior to this region no tail-buds were induced; posterior to it no head buds developed. The region of sharp drop in head formation and rise in tail formation is apparently between the tenth and eleventh segments. Also in this transition zone there were formed a number of buds each of which included at least a portion of an anal segment and, in addition, a part which in some cases showed definite head characteristics.

TABLE I

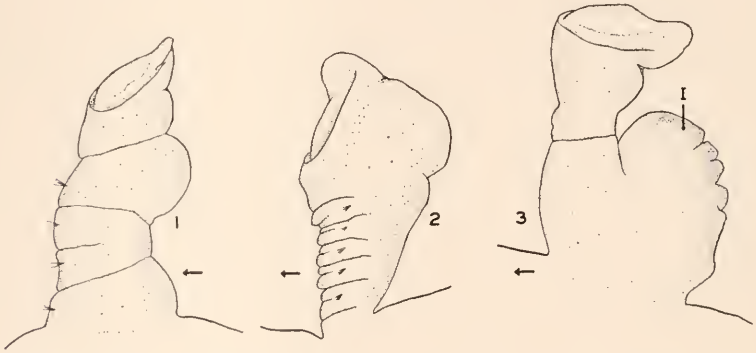
Results of implantation of anterior pieces with anterior end exposed.

Level of Insertion Segments	1–4	5–7	8	9	10	11	12	13–15	Totals	Percentage of Total
Head buds	2	11	11	10	9	2	1		46	26.1
Head-tail buds					2	5		1	8	
Tail-cone buds					3	4	3		10	
Tail buds					3	11	13	13	40	
Indeterminate buds	1	8	8	11	12	10	3	2	55	13.8
No new material	3	5	6	13	4	5	2	8	46	11.6
Died within 10 days	1	4	59	45	27	10	6	15	167	42.0
Implant lost by host	1	4	4	5	5		3	4	26	6.5
Totals	8	32	88	84	65	47	31	43	398	100.0

The various buds exhibiting features of the head, or the tail, or both, are worthy of more detailed consideration. In most cases the description applies to the condition of the bud when last observed before the host died or was killed. Some worms lived only 8 or 10 days after the operation, but did develop buds, while at the other extreme some survived 5 weeks or more.

Both of the head buds of the first group, implants 2–4, were formed at the third segment. In each case the bud consisted of 3 segments. The tip of the implant showed on the ventral side at the base of the bud.

Of the 11 heads formed in the region of segments 5–7, 2 were at the fifth segment. One, implant 5–7, was weakly segmented but included at least 4 segments. The other (Fig. 1), implant 2–4, was made up of 5 distinct segments. Five more of these buds were at the sixth segment. Three, implants 2–3, 5–7 and 5–7, were killed or died within two weeks after the operation without developing segmentation. One, implant 5-7, consisted of at least 3 weakly marked-off segments. The fifth bud, implant 2–4, consisted of 6 segments. The remaining 4 buds were at the seventh segment. One of them (Fig. 2), implant 2–4, was a well-developed bud of probably 7 segments. Two, implants 2–4 and 5–7, were weakly differentiated, but included 6 or 7 segments. The fourth bud, implant 2–4, consisted of 2 setigerous segments followed by an unsegmented region which terminated in a weakly differentiated peristomium.



Arrow points toward anterior end of host in this and all other figures. All figures are camera lucida drawings and × 22.

Fig. 1. Head bud induced at fifth segment. Age, 15 days.

Fig. 2. Head bud induced at seventh segment. Age, 17 days.

Fig. 3. Head bud induced at ninth segment. Age, 17 days. *I*, exposed part of implant.

Of the 11 head buds formed at the eighth segment, 4, implants 1–2, 4–5, 4–5 and 5–6, were weakly differentiated with little or no evidence of segmentation. Two others, implants 2–3 and 5–6, had well-formed peristomial regions but were indistinctly segmented. The remaining 5 buds had well-developed peristomial regions but varied in the number of segments present as follows: two, implants 1–2 and 2–3, included 4 or 5 segments; one, implant 3–4, was made up of only 3 or 4 segments;

one, implant 4–5, consisted of about 3 segments; one, implant 4–5, had formed 7 and probably 8 segments, the same number present in the host anterior to the level of implantation.

Of the 10 head buds formed at the ninth segment, one, implant 3–4, was weakly differentiated. Two, implants 2–4, consisted of only 2 segments (see Fig. 3). One, implant 3–4, was made up of three segments. Two other buds, implants 5–7, included 6 and possibly 7 segments. It may be noted that these buds of 2 or 3 segments, and even possibly the 6 or 7 segment buds, might be interpreted as having their segment-numbers determined by the implant. Two other buds, implants 2–4 and 5–7, were made up of at least 7 and possibly 8 segments. The remaining two head buds, implants 2–3 and 2–4, consisted of at least 8 segments, possibly 9. These last two buds, and probably the two preceding, could best be interpreted as having their segment numbers determined by their position on the host.

The tenth segment was the most anterior level at which tail buds appeared following implants of anterior nerve cord. At this level, however, there also appeared 9 simple head buds. Of the latter, 3 buds, implants 2–4, 5–6, and 5–7, were weakly differentiated and without distinct segmentation. Another bud, implant 5–7, was also weakly differentiated but, unlike the preceding, had 2 small knobs of material on the right side of the peristomial region. During the second and third weeks after the operation, this bud had consisted of an irregular mass of new material the top of which included several rounded elevations. Gradually one of these elevations became the peristomial region and two others became the knobs. This condition existed when the worm was killed 34 days after the operation. Still another bud (Fig. 4), implant 5–6, consisted of possibly 3 segments and included a single knob on the dorsal side of the peristomium. The remaining 4 buds could be considered to have had the numbers of their segments determined by the implants because one of 2 segments followed an implant from segments 2–4 and three buds of probably 5 segments (see Fig. 5) resulted from implants from segments 5–7.

One of the tail buds at the tenth segment, implant 2–4, was weakly differentiated. The other 2 tail buds (see Fig. 6), implants 2–4 and 5–7, consisted of 9 or 10 segments, distinguishable only on the side opposite the implant. In all three of these cases some of the anal segment was lacking on the same side of the bud as the implant. To date there is no evidence contradictory to the idea that the implant is always on the ventral side of the bud. In three other instances, implants 2–4, 5–7, and 5–7, there were unsegmented buds, each with the ventral half of the anal segment missing and with a cone of new material projecting posteriorly into the open region. Figure 9 shows a bud of this type formed at the eleventh segment. This region where part of the anal segment was missing would, in any of these relatively short buds, be

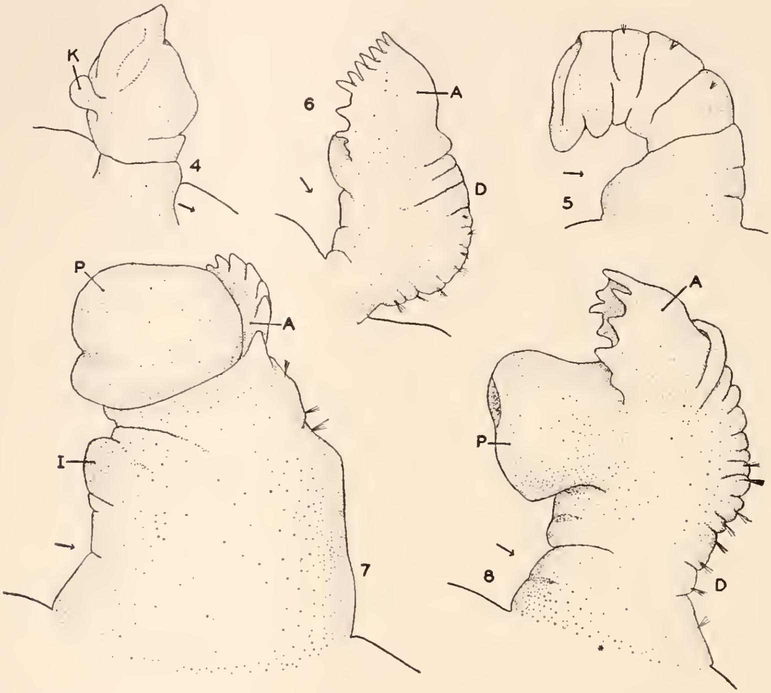


FIG. 4. Head bud induced at tenth segment. Age, 19 days. *K*, knob of new material on dorsal side of peristomium.

FIG. 5. Head bud induced at tenth segment. Age, 18 days.

FIG. 6. Tail bud induced at tenth segment. Age, 22 days. *A*, partial anal segment; *D*, dorsal side of bud.

FIG. 7. Bud induced at tenth segment. Age, 25 days. *A*, partial anal segment; *I*, exposed part of implant; *P*, proboscis-like part.

FIG. 8. Bud induced at tenth segment. Age, 18 days. *A*, partial anal segment; *D*, dorsal side of bud; *P*, proboscis-like part. Asterisk marks basal end of line of demarcation between reddish ventral half and lighter colored dorsal half.

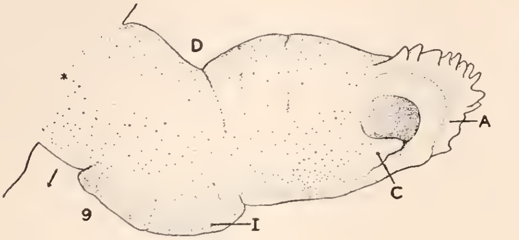
virtually at the outer end of the implant. The cone in each case, therefore, apparently grew from the implant.

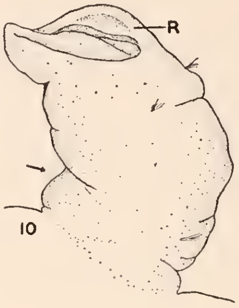
A possible significance of these cones is shown by the remaining two buds, implants 2–4, formed at the tenth segment. One of these buds (Fig. 7) was weakly segmented and terminated in two parts. On the dorsal side was a partial anal segment with about 8 anal cirri. On the ventral side, in the region of the open side of the anal segment, there was a large, rather lobulate region about two-thirds of which exhibited the protractile and retractile movements usually observed in an active proboscis region in *Clymenella.* The behavior of this region, as much as the structure, indicated that it might reasonably be interpreted as a type of partial head bud. The second of these buds (Fig. 8) terminated in several parts, namely: (1) a partial anal segment, dorsally located; (2) a proboscis-like region on the open side of the anal segment and hence ventral; and (3) a small cone of material between the other two parts. In addition there was a fairly definite line of demarcation between a reddish ventral half of the entire bud and a lighter colored dorsal half. There was still a third distinction between the two halves. In the dorsal part it was possible to distinguish 11 or 12 segments with notopodia showing clearly in 8 of them. In the ventral half there were present only 2, or at the most 3, segments. In other words the dorsal half, terminating in a partial anal segment, approximated the plan of the region of the host posterior to the implant bud; the ventral half included about the number of segments which the implant might be expected to determine.

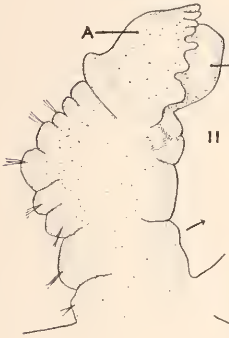
At the eleventh segment the two head buds, implants 1–3, were unsegmented. Of the 11 simple tail buds, all with partial anal segments, 8 had the latter open ventrally; no records were made of this point for the other three. Five of these buds, implants 2–4, 2–4, 5–6, 5–7, and 5–7, were unsegmented and two others, implants 5–7, were too weakly segmented to tell more than that in each there were at least several short segments. Another bud, implant 2–4, consisted of 6 or 7, possibly more, segments. Two other buds, implants 2–4, included at least 7 or 8 segments, evident only on the dorsal side. The last of these tail buds, implant 1–3, was made up of 11 and possibly 12 segments.

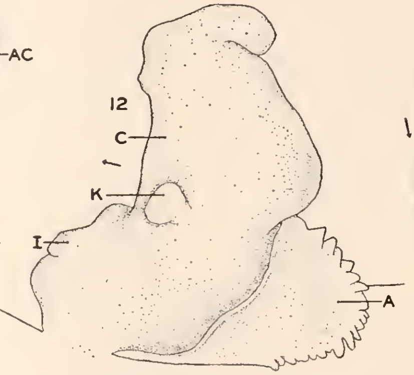
Four buds at the eleventh segment were tails with anal segments open on their ventral sides and with cones of new material projecting into the open region from the implant side. Two of these buds, implants 2–4 and 5–7, showed no color differentiation and were unsegmented. The other two, implants 5–7, were made up of light-colored dorsal halves and reddish ventral halves; one of these was unsegmented, the other (Fig. 9) consisted of possibly several segments.

Of the 5 head-tail double buds at the eleventh segment, 3, implants 2–4, 2–4, and 4–6, were similar in so far as each had a rather pointed projection and a proboscis-like, active region near the open side of its anal segment. One showed 7 or 8 segments dorsally. If there were any segments ventrally, they were few and indistinct. In both the second and the third of these buds the dorsal side showed about 10 segments while the ventral side was occupied mainly by the implant. The fourth double bud, implant 5–7, developed for two weeks in the direction of the type in which a cone of new material occurs near the open side of the anal segment. Then there began a rapid growth of the 'cone' region and a reduction of the anal segment. At the end of 4 weeks this bud was a fairly well-developed head with the anal segment represented only by a knob of material on the dorsal side of the peristomium. Only 5 segments showed in this bud, all of them peripheral to the implant which extended some distance along the ventral side of the bud. The









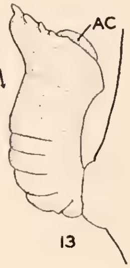


FIG. 9. Bud induced at eleventh segment. Age, 18 days. *A*, partial anal segment; *C*, cone of new material projecting into open side of anal segment; *D*, dorsolateral region of bud; *I*, exposed part of implant. Asterisk marks basal end of line of demarcation between reddish ventral half and lighter colored dorsal half.

FIG. 10. Double bud induced at eleventh segment. Age, 30 days. *R*, ridge (on right side of peristomium) which was derived from anal collar region present two weeks earlier.

FIG. 11. Tail bud induced at twelfth segment. Age, 24 days. *A*, partial anal segment; *AC*, anal cone region.

FIG. 12. Bud induced at twelfth segment. Age, 13 days. *A*, partial anal segment; *C*, large mass of new material growing out from implant region (*I*); *K*, knob of material.

FIG. 13. Tail bud induced at fifteenth segment. Age, 21 days. *AC*, anal cone.

fifth of these double buds, implant 2–4, at first developed a conspicuous cone region with a partial anal collar at its base on the dorsal side. The cone region gradually developed into peristomium and prostomium. A month after the operation this bud (Fig. 10) consisted of 3 or 4 segments with the peristomial region of nearly ordinary appearance except for a conspicuous ridge along its right side. This ridge was derived from the earlier anal collar region.

At the twelfth segment only one head bud, implant 5–6, appeared. It consisted of 5 segments. Of the 13 simple tail buds with partial anal segments, 10 had the opening on the ventral side of the segment; no records on this point are available for the other 3. Of these 13 tail buds, 8,—3 implants from 2–4, 2 from 5–6, and 3 from 5–7,—were unsegmented or so weakly differentiated that it was not possible even to estimate the number of segments present. Four other buds,—implants 1–3, 2–4, 5–7, and 5–7,—included at least 7 or 8 segments. Finally there was one bud (Fig. 11), implant 1–3, which consisted, on its dorsal side, of 10 segments, the same number present in the host posterior to the segment of implantation; ventrally it probably included only a few segments. At the twelfth segment there were also formed 3 unsegmented buds, implants 5–7, each of which included an anal segment region open ventrally and a cone of new material on the ventral side. In 2 of these the cone was comparatively small and extended a little into the open region. In the other (Fig. 12) the 'cone' region was large and made up most of the bud, the anal segment region being located at the base of this larger part and near the host. Unfortunately this worm could not be kept alive beyond the thirteenth day so that it was not possible to observe whether or not further differentiation occurred.

At the thirteenth segment 12 tail buds developed. One of these, implant 2–3, consisted of 10 segments, which were distinct only on the dorsal side of the bud, and had a practically complete anal segment. Another, implant 6–7, was unsegmented but also had a complete anal collar. The remaining 10 had partial anal segments; the opening was on the ventral side in the 7 for which information on this point was recorded. Five of these 10, implants 2–4, 2–4, 2–4, 5–7, and 6–7, were unsegmented. Two other buds, implants 5–7, consisted of at least 6 or 7 segments, probably more. Two buds, implants 5–7 and 6–7, consisted of 9 segments and one bud, implant 1–2, consisted of 10 segments. In these last 5 buds segmentation was weak or lacking on the ventral side.

At the fifteenth segment 1 tail bud and 1 head-tail bud, implants 5–7, were formed. The former (Fig. 13) consisted of 7 segments and had a complete anal segment, rather short on one side. The second bud included at least 5 segments and terminated in a double region. On the dorsal part of the end of the bud was a partial anal collar ventral to which was a contractile region which could be interpreted as a proboscis on the basis of its behavior and general appearance.

Consideration of the indeterminate buds,—those which showed no definite head or tail characteristics,—may be limited to a statement of the five general types which were represented by about equal numbers of buds. These were: (1) buds with slender or rounded tips but showing some evidence of segmentation; (2) unsegmented buds, usually slender, with rounded tips; (3) undifferentiated cones of new material, of various sizes; (4) masses of new material, occasionally with one or two notopodia, which had developed beneath and pushed up all of the implant or a separated fragment of it; and (5) masses of new material, of various sizes and shapes, and frequently including one or more parts such as small knobs, scattered setae, or one or two notopodia.

Table II

Results of implantation of anterior pieces with posterior end exposed.

Level of Insertion Segments	1–4	5–7	8	9	10	11	12	Totals	Percentage of Total
Head buds		1	1					2	2.1
Tail buds							1	1	
Indeterminate buds		3	7	3	3			16	11.3
No new material	1	11	15	11	5			43	30.5
Died within 10 days	6	17	14	18	9	3		67	47.5
Implant lost by host	2	3	6	1				12	8.5
Totals	9	35	43	33	17	3	1	141	99.9

Posterior End of Implant Exposed

As a result of operations in which implants from anterior sources were inserted with their posterior ends out, only 3 buds with definite head or tail characteristics developed. Table II presents the results of 141 operations of this type, 2 of which formed heads and a third a tail. At the sixth segment, the head bud, implant 2–3, consisted of 5 segments, possibly 6. At the eighth segment the head bud, implant 6–7, consisted of 4 or 5 segments. The tail bud at the twelfth segment, implant 2–4, consisted of a rounded mass terminating in a partial anal segment open ventrally.

Of the indeterminate buds, 5 were cones of new material, 2 were small masses of new material with one and two notopodia, and 9 were masses of new material which had pushed up the implants.

Exposed End Unknown

Of the 17 operations for which no record was kept of the orientation of the implant, 4 worms died within 10 days, 8 lived but produced no apparent new material, and 1 host lost its implant. One host, implant 2–3 into segment 3, produced a slender, unsegmented head bud. Another, implant 3–4 into segment 13, formed an unsegmented tail bud with a partial anal segment open ventrally. The remaining two produced only cones of new material.

DISCUSSION

In the data presented in the present report it may be noted that, with a few exceptions, implants were not inserted successfully posterior to the thirteenth segment. Actually 9 implants, only 2 of which produced buds, were satisfactorily inserted in the more posterior region, all of them at the fifteenth segment. Two important factors interfere with successful implantation at these posterior levels. One of these is the marked tendency of *Clymenella* to autotomize posterior segments (Sayles, 1932). Reasonable care prevents breaking of weakly narcotized hosts when implants are inserted into the thirteenth segment. From that level toward the posterior end of a worm, this tendency becomes rapidly greater for several segments. The second factor is the reduction of the coelomic cavity in this same region with the result that the thin-walled, posterior part of the intestine lies almost against the inside of the body wall. In this respect *Clymenella* and other Maldanidae very much resemble members of the closely related family, the Arenicolidae (Ashworth, 1904). This condition increases greatly the task of inserting implants without injury to the digestive tract. This added difficulty, imposed in a region where autotomy readily occurs, results in a very high percentage of unsuccessful operations. Hosts which autotomized at the time of the operation, or soon afterwards, were immediately discarded. Consequently none of these worms appears in the data presented here.

These experiments, first undertaken to learn more concerning the influence of the nervous system on regeneration, soon involved the entire problem of determination in bud formation in *Clymenella*. It has been shown (Sayles, 1939) that, at the thirteenth segment, the nature of the new bud is apparently determined by the host but the orientation of the bud is controlled by the implant. The experiments reported in the present paper substantiate the fact that the implant determines the orientation of the bud and they extend the evidence for this to apply to host segments from the first to the fifteenth. The factors involved in determining the nature of the implant bud may be discussed briefly at this time. Any detailed consideration of the significance of the present data must, however, be postponed until after presentation of comparable data for implants from posterior and mid-body levels.

From a comparison of Tables I and II it is evident that implants possess a definite polarity. Although 26.1 per cent of the 398 implants inserted with their anterior ends exposed produced buds with some definite head or tail characteristics, only 2.1 per cent of the 141 implants with posterior ends out produced similar results. Nearly 30.5 per cent of the cases with posterior ends exposed lived but produced no new material, while only 11.6 per cent of the worms with anterior ends of implants exposed fell in this category. That these differences are great enough to be significant seems to be clear without resorting to detailed analysis of the data. It is, of course, not improbable that occasionally the ends of an implant might have been unwittingly reversed before insertion into the host. Such mistakes might, for example, account for one or more of the 3 cases of definite head or tail structures in the group with posterior ends of implants exposed. At any rate, inclusion of such a reversed implant in this group would, if it affected the results at all, tend to produce too high a percentage value. But even admitting any possible mistakes, the hosts of this group produced an almost negligible number of heads or tails.

It has also been observed that when buds with definite head or tail features appear they organize along the longitudinal axis established by the implant. When, however, the outer tip of the implant bends over and heals into the body wall, new tissue may appear in small amounts beneath the implant and grow out from the sides to some extent. Under such conditions organization is apparently limited to one or two notopodia. It has been found that in regeneration in both oligochaetes (Krecker, 1923) and polychaetes (Probst, 1931 and Faulkner, 1932), the neoblasts migrate to the wound region along the nerve cord. Similarly the new material which contributes to the formation of implant buds apparently migrates along the implant cord. In regeneration in *Clymenella* at anterior levels there is virtually no posterior migration of cells and hence posterior regeneration is limited to wound closure. Whatever the controlling factor in this migration may be, it seems to be retained after separation of a small piece of the nerve cord and neighboring tissues from the rest of the body of a *Clymenella*. The polarity exhibited by implants is, therefore, in accord with ordinary regeneration in this worm.

That the host exerts some influence in determining the type of bud formed is shown by the fact that the relative number of head and tail buds is dependent upon the segments at which the implants are inserted into the body of the hosts. In fact, most of the evidence offered in the present paper points toward the possibility that there is a definite gradient in these worms which brings about the formation of head buds at anterior levels, of mixed structures or heads or tails in the mid-body transition zone, and of tail buds at more posterior segments. This topic, which can be properly discussed only in the light of the knowledge of what occurs when implants from posterior sources are used, will be considered in another paper now being prepared. For the present, therefore, only brief mention is to be made of a few points which indicate that some influence is exerted by the implant, particularly in the transition zone from segments 8 to 12 inclusive. In this mid-body region the number of segments present in induced head buds was the same as the number of host segments anterior to the region of implantation in only 3 (possibly 5) cases out of the 20 in which segmentation was distinct. In all other segmented head buds here the number was less than the corresponding number for the host. Similarly the number of segments in tail buds is usually less than the number in the host posterior to the implant. Clearly, then, it is the common thing for implant buds to include fewer segments than in the corresponding part of the host. In the case of these head buds, however, at least 9 (possibly 12) of the buds possessed the same number of segments as were present in the donor anterior to the source of the implant. The relatively large group of buds in this category seems to be evidence that more than coincidence is involved and that these buds may have had their segment-numbers determined by the implants.

The arrangement of parts in the various types of tail buds offers additional evidence of the influence of the implant. All of the 27 simple tail buds formed at segments 10 to 12 had only partial anal segments. The missing part of this segment was on the ventral side (the same side as the implant) in 21 of these buds, every one for which records on this point were kept. Also in all of the double buds the ventral side of the anal segment was missing. The extra cone, or proboscis-like structure, or peristomial region, developed in each case in line with the end of the implant. Furthermore, in practically all of the simple tail buds and double buds any extensive segmentation was confined for the most part to the dorsal region. In those double buds in which there was evidence of segmentation on the ventral side, the number of segments in that region was small. Although in some cases the taking up of ventral space by the implant itself might have been a contributing factor, this was by no means true in all cases (see Fig. 8). The color difference between the dorsal and ventral halves of several double buds indicates a possible double source of the material for the two parts. To date, attempts to stain implants (with Nile blue sulfate, for example) and to follow the buds induced by them have not met with sufficient success to serve as any real evidence on this point. From the arrangement of parts in these various tail buds it seems that the dorsal sides of such buds organize under the influence of the host and that usually ventral differentiation in these buds may be associated with the implant. At posterior levels segmentation and most of the terminal segment are usually lacking ventrally. In the mid-body region, however, the tail-forming tendencies of the host are somewhat weaker, as shown by the fact that ordinary head buds may result from implants at this level. Here, therefore, the ventral sides of the buds may occasionally be organized by the implant, even though the host determines the fate of the new material which is more dorsal and consequently farther from the implant.

In contrast with these buds in which partial anal segments appeared, there were the numerous head buds, none of which had a terminal segment (peristomium) with one side missing, even though many of these buds were weakly differentiated. Evidence from regeneration indicates that in each of these cases both the source of the implant and the segment of implantation were in regions where head formation was at least possible.

It seems, therefore, that determination of the type of bud formed may be controlled through an interaction between implant and host. Apparently the determining influence of the host is in general dominant at posterior levels. But, of course, even at these segments, the buds are induced and oriented by the implant. In the mid-body region this dominance is not so complete, possibly because this region is a transition zone (as shown in regeneration), at which a worm may form either a head or a tail. The most complete and best differentiated buds, however, are the head buds formed at anterior levels where any influences of host and of implant would in general supplement one another instead of being exerted in different directions.

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THE MEIOTIC CHROMOSOMES OF THE MALE LLAVEIELLA TAENECHINA MORRISON (COCCIDAE) AND THE QUESTION OF THE TERTIARY SPLIT

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The meiotic chromosomes of the male *Llaveiella taenechina* present a new type of observational evidence on the old question of the time of chromosome division. In spite of the overwhelming evidence now accumulated in favor of a multiple strand structure at all stages of both somatic and meiotic chromosomes, there is still disagreement as to the exact number present at any point in the chromosome cycle. Recent reviews of the extensive literature on this question are available (Lorbeer, 1934; Kaufmann, 1936; Geitler, 1938). Suffice it here to say that the vast majority of evidence and of observers supports the idea of a somatic anaphase chromosome at least double in structure which reduplicates in pro- or early metaphase to form a four-parted metaphase chromosome. The last spermatogonal division differs in no way from the preceding one and the meiotic chromosomes are thus theoretically, even if not always visibly, double at leptotene, four-parted at metaphase, and form eight-parted tetrads. The older idea of univalent leptotene chromosomes reduplicating at pachytene to give four-parted tetrads is maintained by few among recent writers, among them Geitler (1938) and Darlington (1937). The persistence of this concept in the face of overwhelming evidence to the contrary stems partially from the reluctance of geneticists to abandon Darlington's precocity theory of meiosis. This hypothesis, as Huskins (1937) has pointed out, has proved stimulating and valuable but its basic assumptions must now be admitted to be untenable. A conservative point of view toward the evidence for multiple strand structure stems also from the technical difficulties in the way of its demonstration; admittedly it cannot be demonstrated for all stages of the cycle nor in all organisms.

In the meiosis of the male *Llaveiella taenechina* the multiple structure of the chromosome is rendered dramatically visible by a unique autonomy of the individual chromatids in their metaphase behavior and their anaphasic movements. Not only is the tertiary split[1] present, but its reality is demonstrated beyond possibility of cytological misinterpretation by the fact that the pairs of half chromatids separated by it proceed in their poleward migration at individually different rates. Two other phenomena of the *Llaveiella* meiosis contribute to the demonstration of the tertiary split—first, the high incidence of asynapsis among the chromosomes, and second, the tendency for the equational halves of the sex chromosome to separate and to divide independently of each other. The possibility of resolving a chromosome optically into its constituent units is obviously increased by subdividing the mass—as in the cases of the asynaptic chromosomes and the separate X chromatids—and by the dissociation of the larger unit by the differential rates of movement among its components.

Material and Methods

Llaveiella taenechina belongs to the most primitive tribe (Llaveiini), and sub-family (Monophlebinae) of the coccid family Margarodidae. The insects were collected in the vicinity of Ixtepec, Oaxaca, Mexico, from their favorite host plant the cascalote, *Caesalpinia coriaria.* Fertilized eggs were transported to New York and the young were raised to maturity in the Columbia University greenhouse. Over 150 males of all ages were available for the present study. Considerable data on the biology of this little-known species have been accumulated and will be published elsewhere. For the purposes of this report I need only say that meiosis in the male occurs during the third and fourth nymphal instars. The testes were dissected out in Allen's modification of Bouin's fixative, sectioned at 4, 5, 6 and 7 micra, and stained with Heidenhain's haematoxylin. Other methods (Benda, Flemming fixations, Feulgen, etc.) were used to check results but the present report is based on the Bouin-haematoxylin preparations.

Acknowledgments

This study was made possible by permission from the Bureau of Entomology and Plant Quarantine of the United States Department of Agriculture to import these insects for scientific study. It is a pleasure to express my thanks to the Bureau and especially to Dr. Max Kisliuk

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[1] The term "tertiary split" refers to the division of the meiotic chromosomes preparatory to the first post-meiotic mitosis—the unused cleavage of the meiotic mitoses. The name "tertiary" derives from the old identification of the plane of contact between paired homologous chromosomes as the primary split (reductional plane of the tetrad in the old terminology), while the split effective in the division of the chromosomes at meiosis (the equational plane of the tetrad) was known as the secondary. The next division plane was accordingly termed the tertiary split.

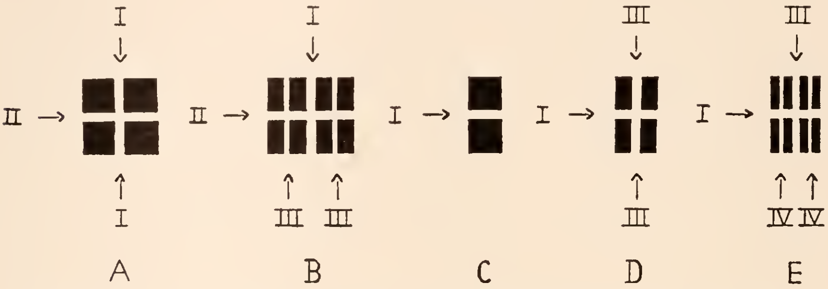
of the New York office for their continued coöperation. I am also deeply indebted to Drs. J. M. and A. C. Baker of Mexico City for continued assistance in the collection and transport of the insects. The field work during 1938 was supported by a grant from the Carnegie Corporation to Professor Franz Schrader of Columbia University.

General Account of Meiosis

The chromosome complement of the *Llaveiella taenechina* ♀ comprises 3 pairs of rod-shaped chromosomes differing in length—one long, one slightly shorter, and one very short pair (Fig. 1). In the the ♂ the shortest chromosome is unpaired giving a complement of 5 (Fig. 2). In its general features meiosis parallels that found in the genus *Llaveia* (Hughes-Schrader, 1931). The chromosomes are the same in number in the two genera; the autosomes, moreover, show very comparable size relations as well as some strikingly similar peculiarities of behavior. The sex chromosomes of *Llaveia*, however, constitute the largest pair,—much longer than either pair of autosomes,—while in *Llaveiella*, they are very short,—much shorter than any of the *Llaveia* set. While of course not conclusive, these relations suggest that the X chromosome of *Llaveiella* is derived from the *Llaveia* X by loss or losses—rather than through any transformation of autosome into sex chromosome. The derivative relationship of *Llaveiella* to *Llaveia* is, moreover, borne out by the detailed study of the cytology of the two forms.

Somatic and spermatogonial mitoses are orthodox both as regards chromosomes and achromatic figure. The meiotic prophase is initiated with the growth and lobulation of the nucleus into 3 separate vesicles proportional in size to the chromosome pairs. In each of the two larger vesicles one pair of the longer chromosomes (autosomes) evolves and passes through characteristic leptotene, zygotene, pachytene, and diplotene stages. In the small vesicle the sex chromosome, visibly split since an early stage, condenses into what appears in cases of maximum contraction to be a bivalent body. The vesicle walls then disappear in situ; each tetrad and dyad concomitantly gives rise to individual half spindle elements. As in *Llaveia*, these half spindles have no central bodies and their fibers diverge in an outflaring truncated cone, instead of converging to a center. At first devoid of common orientation, these individual spindles gradually orient themselves into parallel position, with the chromosomal masses in a metaphase plate arrangement. Figure 3 shows such a typical metaphase—with the chromosome elements at maximum contraction. Figure 4 gives the polar aspect of a similar plate. The two tetrads seem each four-parted—two-parted in polar view—the X chromosome two-parted, with one element only visible from the end. The slightly pointed projections of the chromosome ends, continuous with the half-spindle substance, represent a thin flange or collar of spindle material which seems to be drawn out from the chromosome or to draw out the chromosome sheath as it forms—and do not indicate subdivision of the chromosome. This is clear from the fact that the profile outline remains the same from whatever position the chromosome and its spindle be viewed. Anaphasic movement starts with the compression and elongation of each of the chromosomal elements. An individual, tubular interchromosomal element or stembody, continuous with the chromosome sheath and the boundary wall of the half spindle, grows between the separating chromosome masses—which are progressively constricted like drops in a capillary tube as they pass to the poles (Fig. 5). An interesting variation from the *Llaveia* figure lies in the very early appearance of the cytoplasmic furrow which cuts through the individual stembodies without visibly affecting their structure or position. The sex chromosome divides equationally in this first division and both telophase nuclei in all cases of maximum contraction and synchrony of chromatid movement,—show the same chromosome group of five elements,—two chromatids from each tetrad, one from the X dyad. A short interkinesis with a variable degree of chromosome loosening follows (Fig. 6)—after which the chromosomes recondense. The homologous chromatids then re-associate; and each dyad thus formed gives rise to its own half spindle. The X, usually with some indication of doubleness, produces no half spindle and moves as a passive body to one pole. Division of the dyads proceeds as in first anaphase with the formation of individual stembodies which may later fuse into a single tube during late anaphase.

The foregoing condensed account is based on cells showing the maximum condensation of chromosomes and the maximum synchrony among their elements. It parallels *Llaveia* (*Llaveia bouvari,* Hughes-Schrader, 1931, *Llaveia oaxacoensis,* and an as yet undescribed genus of the Llaveiini, unpublished data Hughes-Schrader) ; is ancestrally rather than derivatively related to the *Protortonia* type (Schrader, 1930) ; and probably represents the basic meiotic pattern for the tribe Llaveiini. Actually, however, in *Llaveiella taenechina* this basic pattern is realized in only a small percentage of the spermatocytes. The variations far outnumber the regular cases. The variations are of four basic types—expressed to different degrees and in different combinations in different cells. As indicated above, these variant procedures involve: (1) chromatid autonomy and differential rates in chromatid separation and anaphasic movements, (2) separation and independent division of the equational halves of the sex chromosome, (3) asynapsis, and (4) the occasional further subdivision of the chromosome along the fourth or quarternary split. I would especially emphasize that all these variants lie within the normal range of meiotic procedure for this species. The evidence for this is convincing. All the variant procedures culminate in a regular reduction of the chromosomes. There is a striking absence of degenerating cells or cysts in the testes of the many males studied; all the visible evidence shows that the variant types are successful in sperm formation. Breeding data further support this conclusion. There is no loss of eggs after fertilization and the percentage of hatching and the viability throughout development is amazingly high. A description of the variant procedures follows.



Text Fig. 1. *A*. Tetrad of *Llaveia*-type with primary (I) and secondary (II) splits. *B*. Tetrad of *Llaveiella*-type, showing an additional, tertiary split (III). *C*. Second division chromosome of *Llaveia*-type, with only the primary split (I) visible. *D*. Second division chromosome of *Llaveiella*-type, with primary (I) and tertiary (III) splits. *E*. Same as *D* with addition of quarternary (IV) split, as occasionally found in *Llaveiella*.

Chromatid Autonomy in Metaphase Behavior and Division Rate

First Division

The tertiary split becomes clearly evident in the majority of first metaphase chromosomes. Only rarely do these show the compact association of two chromatids per chromosome, four per tetrad, of the tribal type (Figs. 3 and 4). Far more frequently the association of the four chromatids in the tetrad is a loose one; they slide upon one another and take up various positions in relation to each other. Thus separated the tertiary split in each becomes clearly evident and, as in Fig. 8, each tetrad is seen to be obviously eight-parted.

The identification of the planes of separation in this and the following figures will be facilitated by reference to Text Fig. 1. In *A* the compact tetrad of the *Llaveia* type is shown. The distinction between the primary and secondary splits (I and II) is, of course, arbitrary and meaningless in cases where crossing over has combined the reductional and equational planes. The identification here used is based on cases of asynaptic chromosomes whose orientation is similar and in which the secondary, and in this case truly equational, split is the one effective in the first division. *B* shows the *Llaveiella* tetrad, subdivided along the tertiary split III—which is thus seen to separate pairs of half chromatids —each pair thereupon assuming autonomy in spindle formation.

Resuming the analysis of Fig. 8,—it is evident that the pairs of half chromatids separated by the tertiary split retain a closer association than do the pairs of whole chromatids. All four pairs of half chromatids may line up parallel to each other in a single row, as in Fig. 8 (a polar view of this arrangement is shown by one tetrad in Fig. 9), or the two quartets may lie at right angles to each other—as shown by both tetrads of Fig. 12 and by one tetrad of Fig. 11. Again the two quartets may lie side by side in two rows giving the compact plate of four elements in end view, as in both tetrads in Fig. 10 and one in Fig. 9. The half spindles reflect to some degree this subdivision and separation of the chromosome elements which seem to give rise to them, but lamination of the half-spindle substance is restricted to the chromosome end of each half spindle. At its distal end the spindle substance of the separate elements seems to fuse into a single flaring, truncated cone (see the stippled end views of cones in Fig. 10).

Even more dramatic evidence for the tertiary split is available in the anaphasic behavior of these chromosomes. This stems from the fact that anaphasic separation and poleward movement of the pairs of half chromatids may proceed at very different rates. Very frequently one pair of half chromatids within a tetrad group starts its anaphasic separation in advance of the others (Figs. 13 and 14). Two factors in chromosome movement may be differentiated here; one, apparently intrinsic to the chromosome, expresses itself in a lengthening of the chromosome elements along the longitudinal axis of the spindle; the second is the formation and growth of the interzonal connective or stembody. If, as appears probable, half-spindle wall and stembody are simply extensions of a chromosome sheath, both effects may be produced by the elongation and thinning of the one tubular sheath with a consequent compression of the chromosomal material. But since changing shape (elongation and thinning) so often precedes any formation of stembody, I am inclined to think that two factors operate here. In the tetrad of Fig. 13 one chromatid pair is dividing precociously; its elements have elongated and it has already formed an individual stembody between them, while the two laterally placed pairs of half chromatids show some change in shape but no stembodies are as yet visible. In the foreground tetrad of Fig. 14 the two paired elements nearest the observer show equal stembody formation between their chromosome masses, while the change of shape factor is precociously expressed in the left-hand chromatid only. In this tetrad, then, we have one chromatid pair precociously elongated and with an initial stembody formation—one chromatid, in the background, which shows neither elongation nor stembody—while the other two chromatids are dividing synchronously with a common stembody and very little elongation of their chromosome elements. Even here, however, the tertiary split is visible in the lower half chromosome, and in its half-spindle component.

The sex chromosome, always clearly bipartite through the prophase and first metaphase, does not clearly disclose its secondary split (homologous with the tertiary split of the autosomes) until the onset of anaphasic movement. Herewith becomes apparent the same tendency to independence of chromatid action as characterizes the autosomes. In the upper cell of Fig. 15 one dividing chromatid of the X shows marked elongation and slight stembody formation; the other, little change in shape and a greater growth of stembody. In the X of the lower cell stembody formation seems equally advanced in the two halves, while the elongation factor alone is differentially expressed. This differential behavior emphasizes the reality of the split, which is often suggested but never positively demonstrable in the more synchronously dividing X chromosomes, such as those shown in Figs. 8 and 14.

In the later anaphase of the first division there usually occurs a fusion of the individual stembodies into a single common tube. Herewith the chromosome elements become massed together and their analysis is rendered difficult. The early differences in time and rate of anaphasic separation persist, however, to different degrees in different cells. Late telophase and interkinetic nuclei vary in the degree of dissociation of their chromosome elements from cases of maximum contraction and cohesion, where only five elements are visible (Fig. 6) to cases in which most or all of the ten half chromatids are distinctly separable optically (Figs. 16 and 34).

Chromatid Autonomy in Second Division

In the second division the effective reality of the tertiary split and the independence of action of the chromatids are even more marked than in the first division, due partly, of course, to the smaller number of elements to be analyzed, but partly also to a real intensification of the differential behavior.

In interkinetic and early second metaphase nuclei so compact an association of chromatid halves as is shown in Figs. 6 and 7, which approximate the tribal type, is very rarely encountered. Even in these nuclei, chosen as examples of maximum cohesion of chromosome elements, it will be noted that one autosomal mass in Fig. 6 and the X in Fig. 7 give evidence of subdivision. The vast majority of interkinetic nuclei show a clear spatial separation of the half chromatids. Similarly the re-association of the chromosomal elements for the second division is but rarely a close one. Instead, there occurs an aggregation of the chromosomes of the interkinetic nucleus into three groups—one comprising the two parts of the X, each of the other two the four derivatives of one autosomal chromosome pair. In this re-association into groups we are confronted with a clear case of chromosome pairing operating among multiple elements. The subdivision of the chromosome does not impair the attraction.

The metaphase stage resulting from this re-association or grouping of half chromatids is very transient and seldom established to the point of a precise parallel orientation or plate-like arrangement of the chromosomes. More frequently the loosely assembled four-parted autosomal aggregations seem to pass at once, as soon as contact between their homologous elements is established, into early anaphasic movement. Their half spindles form while the first steps in chromosome separation are under way. The two halves of the X chromosome, now lying in contact or in close proximity, may take any position in the nuclear area. If in the equator, the sex bivalent sometimes shows an elongation in the direction of the long axis of the developing spindles; if extra equatorial, no such change of shape takes place at metaphase. Whatever its position, no spindle is produced by it and it passes with apparent passivity to one pole, with varying degrees of separation apparent between its two components.

In the anaphase each of the two pairs of half chromatids comprising an autosome forms its own individual spindle, often clearly separable from its mate along the plane of the tertiary split. This autonomy of the chromatid, rather than the chromosome as in the tribal type, in spindle formation and anaphasic movement is established by three lines of evidence. First, the half-spindle element of each half chromatid is initially distinct from that of its mate (Figs. 17 and especially 18, where the two chromatid spindles of each chromosome mass are bent at different angles); second, the stembody may be distinct for each chromatid (note the right-hand chromosome in Fig. 20). Even in cases where the two chromatid stembodies seem to fuse centrally, a double region is often apparent distally as in the left-hand spindle of Fig. 24. Third, and most convincing, the half chromatids of each chromatid separate and pass to the poles at different rates. Again, as in the first division, the two factors of change of shape and of stembody growth may be differentiated. And, as these two factors express themselves to different degrees in the two chromatid spindles of a chromosome, the chromatid autonomy becomes thereby very marked. Thus we may find in a pair of associated dividing chromatids a uniform growth of the stembodies coupled with differing degrees of attenuation of the chromatin masses (see the left-hand chromosome in Figs. 17 and 23). Conversely, differential growth of the two stembodies, coupled with some difference in attenuation, is manifest in both sets of spindles in Fig. 18. As anaphase progresses these differences in rate tend to be maintained or increased. As the two chromatid spindles of a chromosome aggregate elongate, they seem to slide upon one another along the plane of the tertiary division. We have already noted that the stembodies of these two separate chromatid spindles tend to fuse at the equator. Coincidentally each chromatid spindle has undergone a marked elongation; this is evinced by its steady increase in length, decrease in diameter, and compression of the chromatin into flanges along the two walls, both distally and centrally, and in the narrowing and straightening of the half-spindle elements. As this growth in the length of the spindles proceeds, the fusion of the two stembodies noted above becomes complete. The chromatin masses with their separate half spindles are thus brought nearer and nearer together. As long as one pair of separating half chromatids is only slightly in advance of the other, they continue to slide over each other and maintain structural separation (Figs. 22, 23 and 24—the left chromosome aggregate in each case). When the more rapidly moving pair overtakes

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PLATES I–IV

All figures are drawn with Zeiss apochromatic 2 mm. obj. and comp. oc. 20, at table level, with Abbé camera lucida, and are reproduced without reduction.

Plate I

Fig. 1. Diploid chromosome group of female. Oogonial mitosis.

Fig. 2. Diploid chromosome group of male. Spermatogonial mitosis.

Fig. 3. Metaphase I viewed from side. Maximum condensation of chromosomes.

Fig. 4. Polar view of metaphase I.

Fig. 5. Early anaphase I, with maximum contraction and synchrony of chromosome elements.

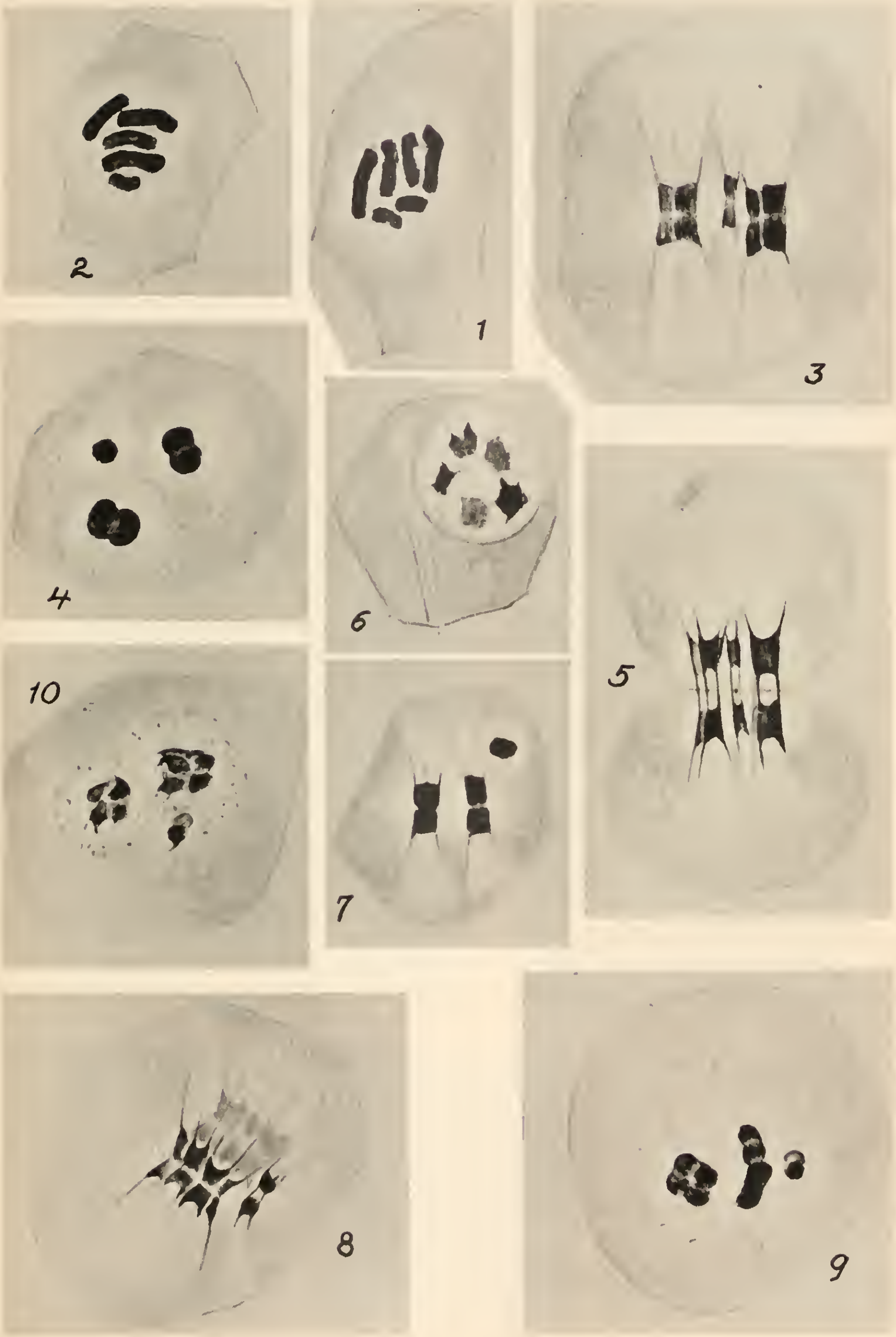
Fig. 6. Interkinetic nucleus—maximum cohesion of elements.

Fig. 7. Metaphase II—maximum cohesion.

Fig. 8. Early anaphase I—with both tetrads showing tertiary split.

Fig. 9. Polar view metaphase I with right-hand tetrad in same position as those of Fig. 8.

Fig. 10. Polar view metaphase I with tetrads in same position as in Fig. 13.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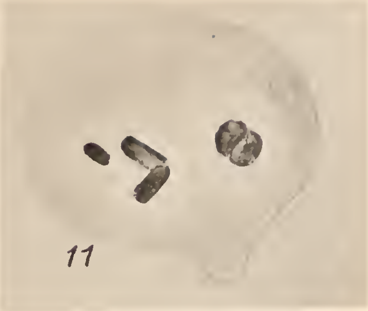
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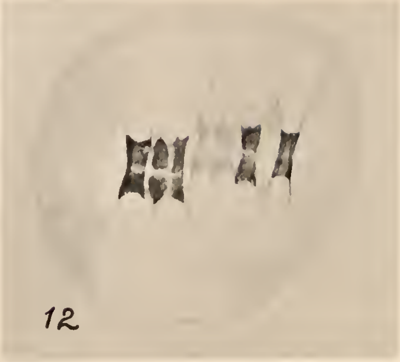
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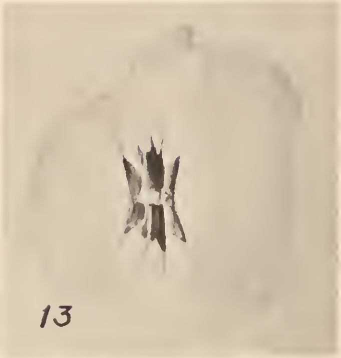
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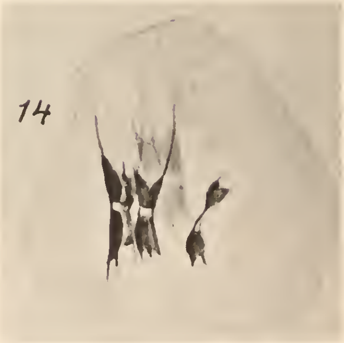
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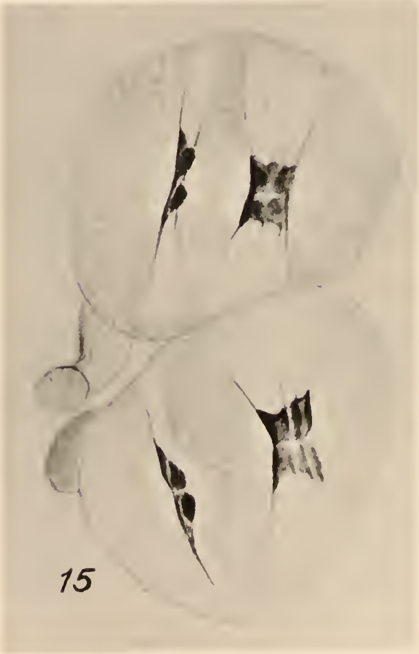
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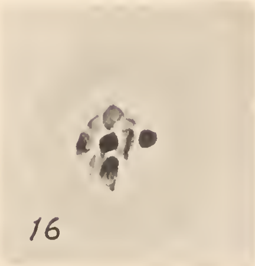
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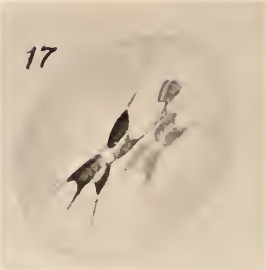
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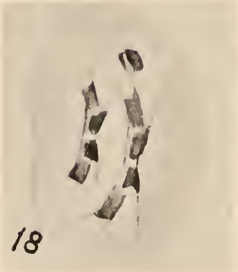
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PLATE 2

the slower ones in the passage to the poles, the two partially separated chromatid spindles tend to fuse completely, giving the curious linear aggregates of four chromosome elements within the single spindle tube seen in the left-hand complex of Figs. 20 and 21, and the right-hand complex in Figs. 22, 23 and 24. This fusion of the two originally distinct chromatid spindles into one never involves the substance of the half chromatids but only the sheath material which from its earliest appearance has appeared to be continuous over half spindle, chromatid and stembody. The sex chromosome is often caught up by one or another of the rapidly growing half spindles; it thus often comes to lie within the substance of one half spindle (Fig. 22). When its two components are closely appressed or lie transversely to the long axis of the spindle (Figs. 20, 21 and 25), a linear aggregate of five chromosome elements within a single tubular sheath is produced. The structure thus formed strikingly resembles the metaphase configuration of the second meiotic division in *Protortonia* males (Schrader, 1931).

Late anaphasic movement involves not only continued linear growth of the stembodies but also a continued elongation and attenuation of each chromosome mass itself. This is not surprising, if as I believe, the tubular sheath so clearly demonstrable in optical sections of the stembody (quite as in *Llaveia* spermatocytes), is actually identical with the chromosomal sheath and this in turn continuous with the outer wall of the half spindle. Elongation of a common sheath would force the chromatin masses to elongate. However, some precocious despiralization of the lagging half chromatids probably enters into the picture also; the equatorially directed end of the chromatin mass often becomes indistinct in stain and fuzzy in outline, with considerable indication of a spirally unwinding chromonema. This may begin while the chromatin

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PLATE II

FIG. 11. Polar view metaphase I with left-hand tetrad in the position shown in Fig. 12.

FIG. 12. Metaphase I, lateral view, with the two quartets of each tetrad at right angles to each other.

FIG. 13. Early anaphase I—only one, the larger, tetrad is drawn. Chromatid spindles, and differential anaphasic rates shown.

FIG. 14. Same as 13—with smaller tetrad in early division phase.

FIG. 15. Same as 13—showing chromatid autonomy in the X components. One tetrad omitted in each cell for clarity.

FIG. 16. Interkinetic nucleus showing at least nine separate chromosome elements.

FIG. 17. Early anaphase II, showing reassociation of autosomal derivatives, and chromatid spindles.

FIG. 18. Anaphase II. Chromatid spindles bent. Different rates of anaphasic movement visible in the different chromatid spindles.

is still within the spindle sheath or tube (Fig. 26) and continues steadily throughout the ensuing stages.

The cytoplasmic furrow cuts through at different stages of these anaphasic changes in different cells—but in no case so precociously as in the first division. As the anaphase proceeds the two stembodies approach each other in the midline and fuse into a single common cylinder (Fig. 26). Continuing growth of the stembody and elongation of the chromosomes bring the latter into contact with the cell wall. The chromosomes buckle under; the stembody may extend stiffly into the cell wall (Figs. 28 and 29), then gradually expands as its central end is cut (Fig. 30), and disappears in situ. During these telophasic stages the half chromatids retain their distinct spatial separation and identity,—one telophasic group revealing four half chromatids, all autosomal (Figs. 29 and 30), the others six, four of autosomal and two of sex chromosome origin (Figs. 27 and 28). Despiralization continues throughout these stages, demonstrating conclusively that each half chromatid represents at least one distinct chromonema. Late telophases show a re-association of these distinctly separated half chromatids or chromonemata into pairs. Nuclei in transition from telophase to the resting condition thus show the chromatin in the form of either two or three linear aggregations which tend to be indistinct in fixation and not positively analyzable as to their contained chromonemata, but whose double structure is usually apparent at one or more points.

PLATE III

Fig. 19. Anaphase II, with one pair of half chromatids showing marked elongation.

Fig. 20. Anaphase II. Chromatid spindles separate in right-hand, fusing in left-hand aggregate.

Fig. 21. Same; with fusion of chromatid spindles complete in left-hand aggregate.

Fig. 22. Same; right-hand aggregate shows quarternary split.

Fig. 23. Stages in fusion of spindles, and marked autonomy in anaphasic rates.

Fig. 24. Same; left-hand aggregate shows quarternary split in one pair of half chromatids.

Fig. 25. Late anaphase II; complete fusion of chromatid spindles forming two linear aggregates.

Fig. 26. Same; despiralization visible in lagging half chromatids.

Figs. 27 and 28. Telophase II, showing continued despiralization. Six elements visible, four autosomal and two X chromosomal.

Figs. 29 and 30. Late telophase II—with early stage in reassociation of half chromatids. These nuclei carry no sex chromosomes.

Fig. 31. Early binucleate spermatid, formed by fusion of products of second division. Shows reassociation of half chromatids into two and three threads respectively.

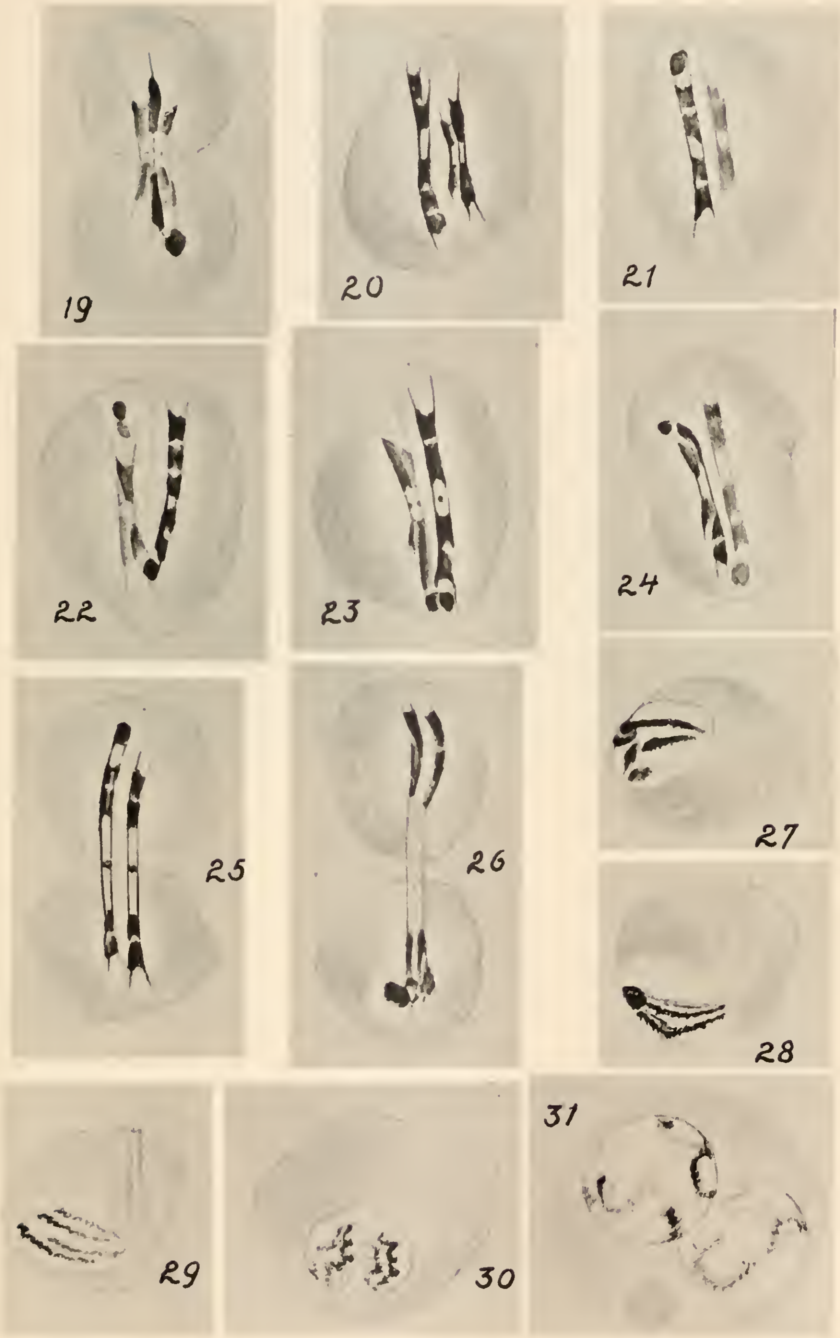
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PLATE 3

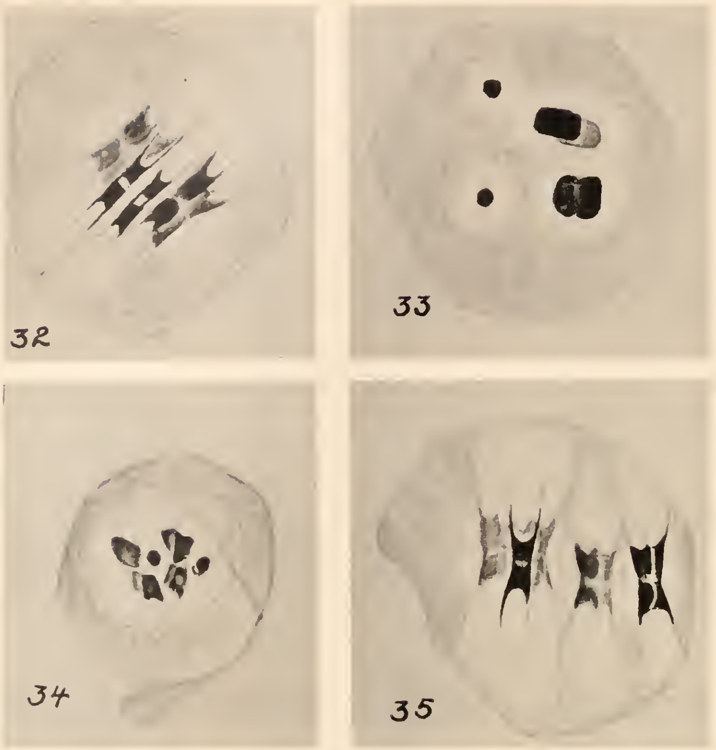

PLATE 4

Fig. 32. Early anaphase I, showing two X chromatids each with its own spindle and dividing independently.

Fig. 33. Polar view of metaphase I with the two X chromatids completely separated.

Fig. 34. Interkinetic or early prophase II with X half-chromatids separated and in one case double.

Fig. 35. Metaphase I—in which the small autosomes, the two right-hand chromosomes, have developed asynaptically and are dividing independently.

Dissociation of the Chromatids of the X Chromosome

This phenomenon may logically be considered as an extreme expression of the chromatid autonomy described in the foregoing section. I separate it to emphasize its bearing on special problems of sex chromosome behavior. The X chromosome, normally double longitudinally throughout the prophase of the first meiotic division, is occasionally found to have separated into its two components. It then appears as two spatially distinct bodies—each measuring half the diameter of the normal X (Fig. 33). This separation of the two chromatids of the X may be traced back, as in the case of the asynaptic autosomes, to the formation of two separate prophase vesicles, in each of which one chromatid of the X evolves. Again, as in the case of the asynaptic autosomes, the subdivision of the mass favors its optical resolution and it is frequently easy to demonstrate that each of these dissociated equational X chromatids is itself double. With the dissolution of the vesicle walls this cleavage, secondary for the X but homologous with the tertiary split in an autosome, becomes very marked. Each bipartite chromatid develops its own half-spindle element and divides independently of the other. In Fig. 33 the wide spatial separation of the independent X chromatids in an early first metaphase nucleus is shown. The vesicle walls have just disappeared, but the nucleoplasm is still visibly demarcated from the cytoplasm. Figure 32 shows the formation of a spindle by each chromatid and the beginning of the anaphasic separation of the half chromatids. It would seem probable that the original dissociation of the X chromatids occurs along the primary split—always so early and clearly evident in the first prophase nuclei. If this be true, the independent division of the dissociated chromatids takes place along the plane of the secondary (= tertiary for autosomes) split. Surely no more final demonstration of the effective reality of this split could be asked. The only alternative assumption is to suppose that the primary dissociation of the X chromatids occurs along the secondary split. Even on this assumption the development of the two chromatids in separate vesicles, the production of individual spindles by each of them, and finally their independent division, form an equally conclusive demonstration of the effectiveness of the tertiary split.

The dissociation and independent division of the chromatids of the X in no way interferes with the normal course of reduction. Telophases of the first division may show a wide spatial separation of the two X components (Fig. 34); but these invariably re-associate during the orientation of the chromosomes for the second metaphase, and come to lie either in contact or very close together (Figs. 17 and 18). Together they then pass to one pole during the ensuing anaphasic movements. In no case out of several hundred second anaphases analyzed, was there a failure of this re-association and reduction.

ASYNAPSIS

As noted above, the first meiotic prophase is initiated, in those cells conforming to the tribal pattern, by a lobulation of the nucleus into three distinct vesicles proportionate in size to the chromosome pairs. The smallest of these vesicles contains the sex chromosome; each of the other two, one pair of autosomes. Within the vesicle the autosomes pass through their synaptic and diakinetic phases. The vesicle walls persist until the chromosomes have reached their maximum contraction, and disappear while the half spindles are forming preparatory to metaphase orientation. Now in a certain percentage of spermatocytes, in all males studied, the members of one or occasionally of both pairs of autosomes develop in separate vesicles and hence completely without any synapsis.[2] Each chromosome develops its own spindle and divides in the first division entirely independently of its mate. The bearing of this phenomenon on the question of the tertiary split lies in the fact that the spatial separation of the two homologous chromosomes, ordinarily so closely associated in the tetrad, renders the quadripartite structure of each first metaphase chromosome unequivocally clear. Figure 35 presents a first metaphase in which the two smaller autosomes have developed asynaptically and are dividing independently of each other. The tertiary split is sharply visible in both. The smaller number of separate elements, compared with the tetrad, and the associated tendency toward a greater separation between them, further preclude any confusion between the tertiary split and that appearance of terminal bifurcation caused by the collar-like flange developed on each half chromatid with the growth of its half spindle.

EVIDENCE FOR A FOURTH SPLIT

There is occasional scattered evidence for still another subdivision of the meiotic chromosome of *Llaveiella*. Thus in a telophase following the independent division of dissociated X chromatids, a clear bifurcation of one of the X components was encountered in three cases (Fig. 34, right-hand X). In the autosomes also a fourth split is occasionally clearly demonstrable. Not infrequently, during the differentially progressing anaphasic movements in the second division, one notes a partial subdivision of one pair of the half chromatids. In several nuclei this division was so complete and so emphasized by the different poleward rates of the quarter chromatids involved as to offer quite conclusive evidence for the reality of the fourth split. Thus in the right-hand spindle in Fig. 22 six well separated chromosome elements are visible. The spindles of the two pairs of separating half chromatids of one autosomal group have here coalesced into a single tube; one pair of the contained half chromatids has divided again, and its halves are moving poleward at different rates,—thus giving six elements in a linear aggregation. Upon careful focusing the two terminal elements in this chain appear to have greater mass than the others; it is thus probable that it is the slower, more centrally placed pair of half chromatids which has redivided. However, all these elements are so small that attempts at accurate measurement proved futile. That these six elements are all derivatives of the one pair of autosomes involved is clear from the presence of the two X chromatids in the half spindle of the other autosomal aggregate. Again in the left-hand spindle in Fig. 24 one pair of the separating half chromatids is again clearly subdivided, with one pair of derivatives well in advance of the other in its poleward movement. In this case the spindles of the two chromatids involved are fused only in the stembody region, so that it is here the more precociously dividing chromatid that shows the fourth split.

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[2] As in *Llaveia bouvari,* it is the smaller pair of autosomes that shows a regularly high incidence of asynapsis. In *Llaveiella* the number of spermatocytes showing asynapsis of this pair ranges from 2.4 to 8.7 per cent. A comparative study of the asynaptic phenomena in three species of Llaveiini and their significance for theories of meiosis is under way at present.

Discussion

Number of Chromonemata per Chromosome

What is the bearing of these divisions of the chromosome along the tertiary and even quarternary splits on the question of the actual duplication of chromonemata, and their number per chromosome at a given point in the meiotic cycle? It is clear that the divisions here described involve the whole chromosome—sheath, matrix and contained chromonemata. The techniques employed do not permit a differentiation of chromonemata within the chromosome unit at all stages. In the telophase of the second division, however, each visible subdivision of a chromosome presents the appearance of a coiled thread, progressively elongating by despiralization. In these stages a one-to-one relation between the chromosome unit as visibly differentiated throughout the cycle, and its chromonemal content is indicated. But even without this evidence, it is obvious that the complete cleavages of the chromosome into chromatids, half chromatids and quarter chromatids as seen in the

Llaveiella material do give us a minimum limiting value for the number of chromonemata present. Obviously each subdivision must contain at least one chromonema. The meiotic chromosome thus comprises at least four chromonemata at metaphase—at least two in anaphase and telophase. The occasional clear separation along the quarternary split would imply that this minimum value is below the actual value—that the metaphase chromosome is at least eight-parted, the anaphase and telophase chromosome, four-parted. Such values would agree with the findings of Nebel (1932, 1933*a* and *b*, 1936, 1937), Nebel and Ruttle (1936, 1937), Stebbins (1935) and Goodspeed, Uber and Avery (1935). In *Llaveiella,* however, the evidence for the regular occurrence of these higher values is not conclusive. The quarternary split, even if we assume it to be a constant feature, is here very rarely followed by a complete cleavage of the chromosome, and hence is only rarely demonstrable. For a minimum value of two chromonemata per chromosome at the lowest point in the cycle, on the other hand, the evidence seems incontrovertible, and for the following reasons. Cleavage of the chromosome along the tertiary split occurs in the majority of the spermatocytes. Moreover, the completeness of the cleavage and the actual movement of the chromosome elements along its plane preclude cytological misinterpretation. Darlington condemns as unreliable much of the earlier evidence for the tertiary split because it depends on the detection of longitudinal doubleness in a cylinder which he claims is less than one half the wave length of the light used. This objection ignores, as Kaufmann (1936) has pointed out, all the evidence from widely diverging chromosome ends, split satellites, and end views of chromonemata. Moreover, it certainly cannot apply to the *Llaveiella* case, for here we are not dealing with the admittedly difficult differentiation of coiled threads intimately associated in a common cylinder. Rather the *Llaveiella* evidence rests on complete cleavages of the whole chromosome into units whose spatial separation and individual behavior preclude optical illusion in their interpretation.

Chromosome Pairing

These data also permit certain conclusions on the forces involved in chromosome pairing. The mutual attraction of homologous chromosomes prior to their reductional separation is here demonstrated to be quite independent of the subdivision of the chromosome, and hence of the multiplicity of chromonemata. In the interkinetic nucleus ten spatially separate and distinct half chromatids may be distinguished. At the prophase of the second division these units re-associate according to their derivation into three groups—one of which contains the two derivatives of the X chromosome, each of the other groups the four derivatives of one pair of autosomes. The force that brings these together is clearly independent of the subdivision of the original chromosomes.

Furthermore the data demonstrate once again that this association is also independent of synapsis and of the formation of chiasmata. This is shown by the fact that the two chromosomes constituting one or both pairs of autosomes may pass through the meiotic prophases in separate vesicles without any possibility of synapsis or of crossing-over, may each divide independently of its mate—and still the derivatives of the two homologous chromosomes will come together in brief contact prior to their reductional separation in the second division. This independence between the attraction factor on the one hand and synapsis and chiasmata formation on the other obtains also in *Llaveia* (Hughes-Schrader, 1931) and reaches complete expression in *Protortonia* (Schrader, 1931).

Furthermore, this attraction operates between strictly equational derivatives of one chromosome, as well as between derivatives of homologous chromosomes. This is attested by those cases in which the two chromatids of the X chromosome have been completely separated during the first division. It will be recalled that occasionally the equational halves of the X evolve in separate prophase vesicles, each develops its own spindle, and divides quite independently of its mate. In such cases the half chromatids of the X may be widely separated in the interkinetic nucleus, yet they always re-associate prior to the second division. It may be added, although it raises another question, that the passivity of the X in the second division, expressed in its failure to produce a spindle, is also seen to be independent of its subdivision.

Evolutionary Relationships

The tribe Llaveiini is recognized on both cytological and more orthodoxly systematic grounds (Morrison, 1928; Schrader, 1930; Hughes-Schrader, 1931) as the most primitive unit of the most generalized subfamily and family of the Coccidae. The *Llaveiella* case supports this conclusion and in addition throws some light on the evolutionary interrelationships within the tribe Llaveiini. Of the three genera of this tribe thus far studied—*Llaveia, Protortonia,* and *Llaveiella, Llaveia* presents the basic or ancestral type of male meiosis, primitive in the high degree of retention of synapsis, specialized in its compound chromosomally derived division figure, and in the incidence of the asynaptic habit. *Llaveiella* retains this basic pattern sufficiently to demonstrate a close but derivative relationship. It adds (1) an increased expression of the asynaptic habit, (2) chromatid, rather than chromosome, autonomy in the formation of the compound chromosomal division figure and in the anaphasic movements of the chromosome elements, (3) the subdivision of the chromosomes along the tertiary and occasionally even the quarternary split, and (4) a tendency for the separation and independent division of the equational halves of the X chromosome.

Without question the genus *Protortonia* presents the most highly specialized conditions in the group. I have elsewhere discussed its probable derivation from a *Llaveia*-like pattern (Hughes-Schrader, 1931). The *Llaveiella* case is of especial interest in this regard because it suggests some of the changes that may have been instrumental in the evolution of the *Protortonia* meiosis.

Take first the question of asynapsis. Incipiently developed in *Llaveia*, where the two shorter autosomes evolve in separate vesicles and divide independently in a small percentage of the primary spermatocytes, this procedure is definitely more marked in *Llaveiella*. Here the asynaptic habit characterizes the smaller pair of autosomes in a larger percentage of the cells, and occasionally also involves the other pair of autosomes. In *Protortonia* this asynapsis is completely established as regular procedure for all chromosomes—the only remnant of synaptic behavior being the retention of a single vesicle for one pair of autosomal derivatives in the first prophase. The complete expression of asynapsis in this related genus supports the idea that its partial expression in *Llaveia* and *Llaveiella* has also a genetic basis, and is significant for the direction of evolution within the group.

Again, the complete subdivision and separation of the equational halves of the first meiotic prophase chromosomes, so unique a feature of the *Protortonia* cycle,—is foreshadowed in *Llaveiella*. In the latter case only the X chromosome is involved, and that only in a very small number, approaching 1 per cent, of the cells. A unique feature of the *Llaveiella* case is the independent division of the separated X chromatids —a feature probably correlated with the strong expression of the tertiary split (here effective in division) in *Llaveiella* and its absence in *Protortonia*.

To me the most suggestive application of the *Llaveiella* data to the problems of the *Protortonia* meiosis involves the possible origin of the strange linear aggregate of chromosomes in the spindle of the second division of the latter genus. It will be recalled that in *Protortonia* the five chromosomes of each interkinetic nucleus formed short chains of two and three elements each. These chains then become appressed together, the chromosomes of one slipping into the interstices of the other chain in such a way that there results a single linear aggregate of five elements lying within a common tube. From the terminal chromosomes of this chain delicate half spindles are formed. Anaphase movements then separate the chain into one group of three, always including the X chromosome, and one of two chromosomes. The tubular stembody involved in this anaphasic movement, together with the half spindles, demonstrate its essential likeness to the meiotic spindles of *Llaveia* and *Llaveiella*.

I would suggest that the two short chains formed from the interkinetic chromosomes in *Protortonia* are to be interpreted as precocious anaphase spindles; that the "fibers" (tubes) connecting them are precociously formed interzonal connectives (stembodies); and finally, that the different distances separating the chromosomes within the two chains represent differences in rate or time of start of the anaphasic chromosome movements. Ordinary prophase and metaphase would thus be considered as entirely eliminated in the second division in *Protortonia* (in this respect compare *Llaveiella* where such a process is well under way). That one of the two chains in *Protortonia* often contains three chromosomes means simply that the X (more or less passive in the second division) has been caught up in one spindle as it forms,—and may thus occupy either a terminal or a central place within the group. The passivity of the X, unless or until caught up into one of the developing spindles, is shown in Schrader's figures 26 and 27. The analogy with *Llaveiella* may be pursued further. Just as in *Llaveiella* the separate spindles of the two chromatids, whose chromosomal elements are separating at different rates, often tend to slip over one another and actually to fuse into a single tubular spindle containing four (or five or six if the X be involved) chromosome elements in linear order—so, too, in *Protortonia* the single chain of five may be formed by the fusion of the two differentially developed precocious anaphase spindles. In *Llaveiella*, of course, the chromatid is the unit in the differential development of the spindles, while in *Protortonia* the whole chromosome assumes this rôle. Further support for this concept of differential anaphase rates in *Protortonia* is found in the first division. It will be recalled that here the separate equational halves of the X and of each of two of the autosomes do actually form their individual spindles at slightly different times and rates. The behavior of the other two chromosomes in the first division in *Protortonia* is not so easily analyzed on this hypothesis. The four equational halves of this pair of chromosomes evolve in a single, instead of in separate vesicles, and they take up a linear alignment within the vesicle before any spindle forms between or involving them. When the spindle does form, however, just as the vesicle walls disappear, it shows the same fundamental structure and arrangement and behavior of its chromosomal elements as does the linear aggregate in the second division of *Protortonia* and *Llaveiella*. This linear alignment preliminary to spindle formation emphasizes the reality of that extrachromosomal force postulated by Schrader in his analysis of the *Protortonia* figure. This force is expressed in the elongation and terminal attenuation of the prophase vesicles and in their convergence to polar centers (Schrader's figures 13 and 14), and would seem to provide an adequate causal factor for the linear aggregation of the chromosome units. That this force is interacting even here with one of intrachromosomal nature is indicated by the differential rate of spindle formation and anaphasic movement characterizing the first division chromosomes. In the formation of the single linear aggregate of the second division, and in the similar configurations in *Llaveiella,* on the other hand, there is a lessened expression of the extra-chromosomal force (thus no vesicle pressure, no convergence to polar centers), and the intrachromosomal forces are hence more obvious. Even here, however, the extrachromosomal polarizing force may well be the cause of the close appression and actual fusion of the two originally separate anaphasic spindles. The balance between the intra and extrachromosomal factors is thus seen to be characteristically different in the three genera available for comparison. In *Llaveia* the extrachromosomal force is weak (witness the complete absence of polar centers, the delay and incomplete metaphase orientation of spindles); the intrachromosomal forces making for linear aggregations (chromatid autonomy and differential anaphasic rates) are also not active; the resultant is the complete absence of linear arrangement. In *Llaveiella,* too, polarization is weak (the fusion of spindles being its only obvious expression), but the divergent rate factor is strong. The result is frequent but variable formation of linear aggregates. Finally in *Protortonia* both factors are operative to a high degree, with a consequent constant formation of linear aggregates.

The Normal Range of Variation in the Meiotic Mechanism

The unparalleled diversity of meiotic phenomena presented by male coccids forces us to keep our working hypotheses of the mechanisms involved flexible. The retention by the females in each species thus far studied of an orthodox meiotic behavior, while the males of the different groups have developed their amazing range in method, is evidence that we are dealing here with successful, workable modifications of the common mechanism—not with anomalies or abnormalities lying without the law. The justness of this point of view receives striking confirmation in the case of *Llaveiella taenechina.* Here within a single individual sister cells vary in such supposedly basic attributes as the presence or absence of synapsis, the occurrence or omission of chiasmata, in the rate and incidence of anaphasic movements, in the time relations of chromosome division. Indeed individual chromosomes within a single cell are seen to vary in respect to these important particulars. And yet in all the variants a normal and regular reduction is consummated. It seems, therefore, that these variant meiotic procedures, going on side by side in the same testes with the tribal type of meiosis, and in the same species with a perfectly orthodox meiosis in the female line must be admitted as falling within the normal range of variation of the meiotic mechanism. The evidence they offer is applicable to and must be taken into account in any general theory of meiosis.

SUMMARY

1. The diploid chromosome set of the female *Llaveiella taenechina* comprises three pairs of rod-shaped chromosomes distinguishable by size. The shortest one is unpaired in the male, giving a diploid set of 5.

2. The chromosome behavior and achromatic figure are normal in the female cycle and in the male somatic and spermatogonial mitosis. Meiosis in the male conforms to the *Llaveia* type, but only a minority of the spermatocytes adhere strictly to this scheme. The majority of the cells show different combinations of four major types of variation, all of which are successful in sperm formation. The variant procedures may be summarized as follows.

(*a*) Chromatid autonomy. The chromosomes are subdivided along the tertiary split. Each pair of half chromatids produces an individual spindle. Anaphasic movements may start at different times and proceed at different rates in the four chromatid spindles. In the second anaphase the two chromatid spindles of an autosomal group may fuse to form a linear aggregate of chromosomal elements in a single tubular spindle.

(*b*) The complete dissociation of chromatids. The two chromatids of the X chromosome may evolve in separate prophase vesicles and divide independently of each other in the first division. The re-association of their derivatives and their common passage to one pole in the second division is not thereby affected.

(*c*) Asynapsis. Each member of one or both pairs of autosomes may evolve in a separate prophase vesicle, without synapsis and chiasma formation, produce an individual spindle, and divide independently of its mate in the first division. Chromosome pairing for the second division is not thereby affected.

(*d*) Quarternary split. Occasionally one or more chromosomes may be further subdivided along the fourth or quarternary split.

CONCLUSIONS

1. The multiple structure of the meiotic chromosome is established. The complete cleavage of the chromosome along the tertiary split gives a minimum value of two chromonemata per chromosome at the lowest point in the cycle.
2. The force effective in chromosome pairing is independent of the subdivision of the chromosome and hence of the multiplicity of chromonemata.
3. The meiotic association of homologous chromosomes is independent of prophase synapsis and of chiasma formation.
4. Chromosome pairing operates between strictly equational derivatives, as well as between those from different homologous chromosomes.
5. The passive behavior of the sex chromosome in the second division—its failure to produce a spindle and undergo anaphasic separation,—is independent of its subdivision.
6. The data support the theory that at least two forces operate in anaphasic movement, one intrinsic to the chromosome, and one expressed in stembody growth.
7. Linear aggregates of chromosomal elements in a single tubular spindle may arise by fusion of individual chromatid spindles.
8. The evolutionary relationships within the tribe Llaveiini are discussed with especial reference to the meiotic division figure of *Protortonia*.

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DESCRIPTION OF A NEW SPECIES OF MARITREMA NICOLL 1907, MARITREMA ARENARIA, WITH STUDIES OF THE LIFE HISTORY

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HISTORICAL

The genus *Maritrema,* established by Nicoll in 1907, includes a number of small distomes, most of them intestinal parasites of shore birds. The most characteristic morphological feature of the group is the ring-like arrangement of vitellaria, which almost completely encircle the body posterior to the testes. Other features of diagnostic value are the approximately equal size of the suckers; the exit of the male duct at a small papilla located near the acetabulum, and continuous with a well-developed cirrus sac; and restriction of the voluminous uterus to the posterior third of the body.

The species of *Maritrema* described to date are as follows:

M. gratiosum—Nicoll, 1907.
M. lepidum—Nicoll, 1907.
M. humile—Nicoll, 1907.
M. linguilla—Jägerskiöld, 1909.
M. subdolum—Jägerskiöld, 1909.
M. nicolli—Travassos, 1921.
M. pulcherrima—Travassos, 1929*a, b.*
M. sachalinicum—Schumakowitsch, 1932.
M. acadiae—Swales, 1933; Ciurea, 1933.
M. rhodanicum—Carrère, 1936.
M. ovata—Rankin, 1939*b.*

An apparently new species of *Maritrema* was recovered from the intestine of the black-headed gull by Rothschild (1938), who indicated in a footnote that a description of the form was forthcoming.

M. obstipum (Van Cleave and Mueller, 1932; Mueller, 1934—Syn. *Microphallus obstipum*), *M. medium* (Van Cleave and Mueller, 1932; Mueller, 1934—Syn. *Microphallus medium*), and *M. nettae* (Gower, 1938), previously in the genus *Maritrema,* were removed by Rankin (1939*b*) to a new genus, *Maritreminoides.*

Description of Maritrema arenaria, N. Sp.

Examination of the intestines of a number of ruddy turnstones (*Arenaria intrepres morinella*) revealed a consistently heavy infection with small microphallid worms of an hitherto undescribed species of *Maritrema.* While other flukes have been found in the turnstone (Lebour, 1909; Rankin, 1939*a*), they occur in relatively small numbers. It seemed, therefore, appropriate to designate the new species of *Maritrema* as *M. arenaria.* Type and cotypes have been deposited in the United States National Museum, Washington, D. C., numbered 9293 and 9294 respectively.

M. arenaria has the characteristic tongue-shaped outline, measuring 0.858 mm. in length × 0.380 mm. in greatest width in the living animal. These measurements were obtained from a well-developed adult worm under slight pressure of a cover glass. Like all flukes of this type, the animal exhibits considerable capacity for contraction and elongation. The body is sparsely covered with cuticular spines from its anterior end posteriorly to the level of the middle of the ovary. The acetabulum is well-developed, practically circular in outline, and located in the midbody region.

Excretory System

The excretory system is of the "Mesostoma" type (Rothschild, 1937) +, that is, each of the main collecting tubes divides into an antero-lateral and a postero-lateral branch in the region of the ventral sucker—with a flame cell pattern of $2[(2+2)+(2+2)]$, conforming to the fundamental pattern for the family *Microphallidae* (Faust, 1932). The main collecting tubes are continuous posteriorly with a Y-shaped excretory vesicle, emptying through a posteriorly terminal excretary pore.

Digestive System

The oral sucker is subterminal and roughly circular in outline, surrounding the mouth. The contracted prepharynx is short, equal in length to the pharynx, leading into a relatively long oesophagus. One-third of the body length from the anterior end, the oesophagus branches to form intestinal crura which extend to the anterior border of the testis.

Male Reproductive System

The oval testes are entire, symmetrical, and approximately one-third of the body length from the posterior end. Their long axes are di-

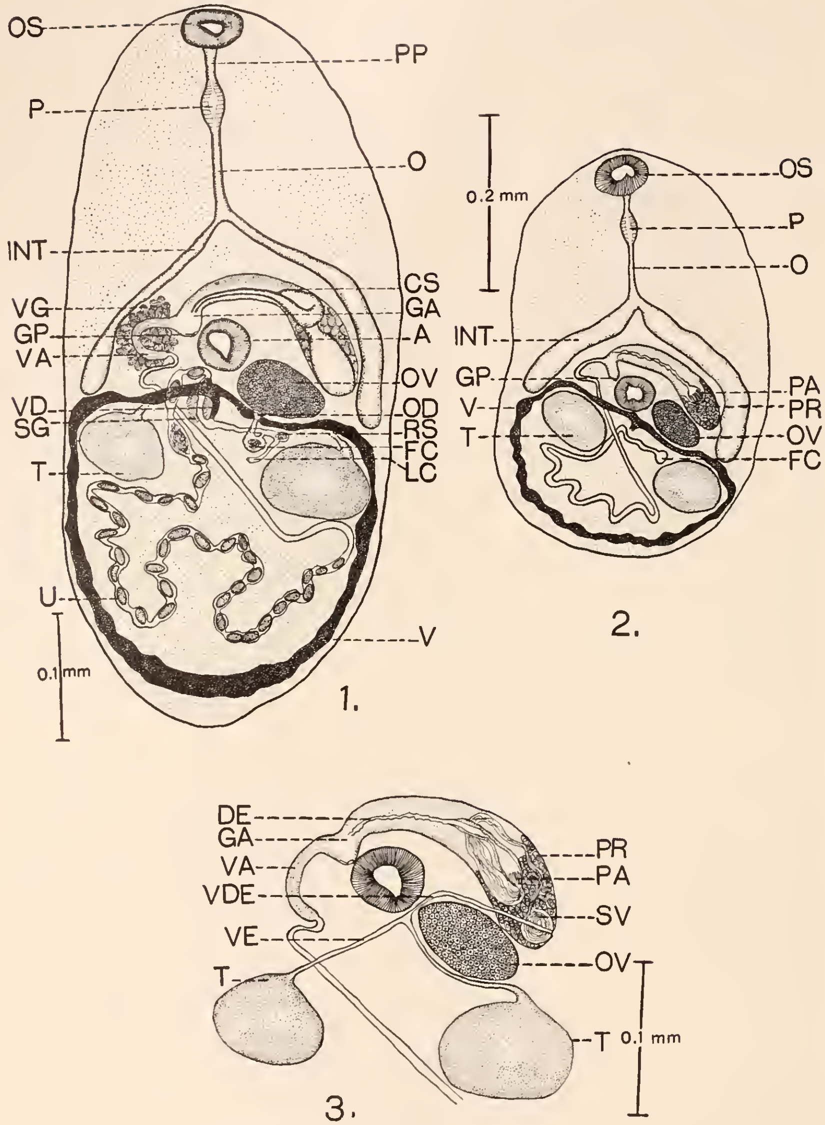
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PLATE I

All drawings made with camera lucida. Abbreviations used: *A*, acetabulum; *CS*, cirrus sac; *CW*, cyst wall; *DE*, ductus ejaculatorius; *FC*, fertilization chamber; *GA*, genital atrium; *GP*, genital pore; *GPA*, genital papilla; *INT*, intestine; *LC*, Laurer's canal; *O*, oesophagus; *OD*, oviduct; *OS*, oral sucker; *OV*, ovary; *P*, pharynx; *PA*, pars prostatica; *PP*, prepharynx; *PR*, prostate glands; *RS*, receptaculum seminis; *SG*, shell gland; *SV*, seminal vesicle; *T*, testis; *V*, vitellaria; *VA*, vagina; *VD*, vitelline duct; *VDE*, vas deferens; *VE*, vas efferens; *VG*, vaginal gland; *U*, uterus.

rected transversely. The vasa efferentia unite close to the left border of the ovary, to form the vas deferens, which extends dextrally along the anterior border of the ovary, crosses the cirrus sac obliquely near its proximal end, and enters the sac subterminally at its antero-lateral border. The cirrus sac is long and curved, extending from its enlarged proximal end, located at the right between the ovary and the right intestinal ramus, in the form of an arch applied closely to the anterior border of the acetabulum. Its distal end joins the genital atrium located on the left border of the acetabulum. The seminal vesicle is well-developed and coiled on itself in the bulbous proximal end of the cirrus sac, as indicated in Fig. 3. Prostate gland and pars prostatica are present, and associated with a slightly coiled ductus ejaculatorius, which empties into the genital atrium by a relatively small genital papilla.

Female Reproductive System

The ovoid ovary lies immediately anterior and slightly mesial to the right testis, with its long axis in a transverse plane, and its broader end toward the mid-line. The short oviduct, leading posteriorly from the caudal border of the ovary near its mesial end, widens to form a spherical chamber, commonly filled with mature sperms. This chamber we believe to be a fertilization chamber. It is joined to the proximal end of the oötype subterminally by a short duct. Dextrad to the point of union with the fertilization chamber, the oötype ends blindly in a slight enlargement, also commonly filled with sperms. This enlarged portion of the oötype we interpret as the receptaculum seminis. Laurer's canal arises from the caudal side of the receptaculum seminis, curving posteriorly toward the mid-line, and ending in a dorsal pore. From its point of union with the fertilization chamber, the oötype extends in a transverse plane dorsal to the oviduct, and receives the common vitelline duct in the mid-line of the body. The oötype then turns sharply anteriorly, and is seen to be surrounded by faintly staining glandular cells, apparently representing shell glands. Continuous with the oötype, the uterus arises near the posterior border of the acetabulum and then follows an undulating course posteriorly along the mesial border of the left testis. In the posterior region of a mature worm, the uterus forms nu-

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PLATE I

FIG. 1. Adult *Maritrema arenaria,* dorsal aspect, whole mount, standard alum haematoxylin.

FIG. 2. Metacercaria of *M. arenaria,* dorsal aspect, whole mount, Lynch's precipitated borax carmine. (Slightly distorted specimen.)

FIG. 3. Male genital organs and associated structures of an adult *M. arenaria,* dorsal aspect.

merous egg-filled coils, which eventually extend around the caudal edge of the right testis, and then continue diagonally to a point anterior to the lateral end of the left testis, where it joins the metraterm. The metraterm, or vagina, is curved, has unusually thick walls, and is surrounded by a dense mass of deeply staining cells which appear to be glandular. These cells we interpret as vaginal glands (Figs. 1, 7). Anteriorly the vagina leads into the genital atrium. The vitellaria, characteristic of this genus, form an almost complete circle, lying just inside the borders of the posterior end of the body, and inclosing both testes. The left and right vitelline ducts unite a short distance posterior to the acetabulum in the mid-line of the body.

Comparison with Previously Described Species

The species known and described up to 1939 were reviewed by Rankin in his paper of that year. The eleven forms of *Maritrema* listed by him may be distinguished from *M. arenaria* by the following easily noticeable differences:

M. lepidum, M. humile, M. linguilla, M. nicolli, and *M. pulcherrima* —by their shorter intestinal crura; *M. gratiosum* and *M. acadiae*—by the median to slightly dextral position of their ovary; *M. ovata*—by the more median position of ovary *and* testes; and *M. subdolum, M. sachalinicum,* and *M. rhodanicum*—by disparity in the size of the two suckers.

The Metacercaria

The metacercarial stage occurs in the common barnacle, *Balanus balanoides.* These cysts are found in practically all barnacles on rocky shores visited by ruddy turnstones. In regions where the surf is heavy, it is noticeable that turnstones are absent, and the barnacles consistently small and uninfected. Barnacles from Norman's Woe and Chebeague Island, Maine, were found to be heavily infected, which fact makes it seem probable that the barnacles all along this coast are parasitized.

The thin-walled, spherical cysts occur most numerously around the gut of the host, but in cases of heavy infection—that is, 600–1,000 cysts in a single barnacle—they are found in every part of the body except the appendages and the interior of the gut.

The cysts are yellow in color, and show clearly the curled-up metacercaria, with gut crura and oesophagus. A large cyst measures ca. 0.328 mm. × 0.343 mm., and when opened by dissection with fine needles, liberates a metacercaria ca. 0.576 mm. long × 0.360 mm. in greatest diameter, with anterior and posterior suckers of about the same diameter, 0.055 mm.

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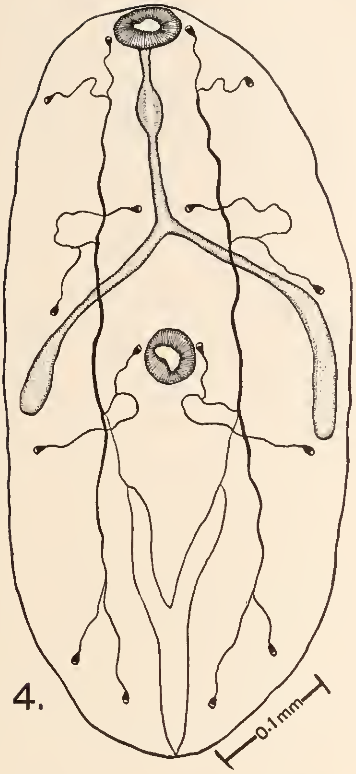

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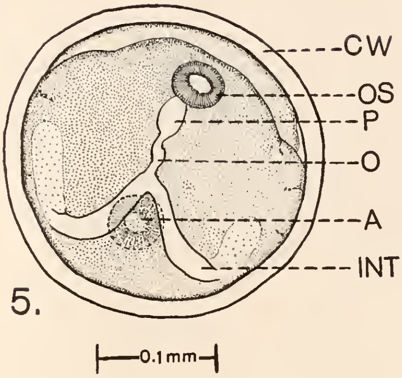
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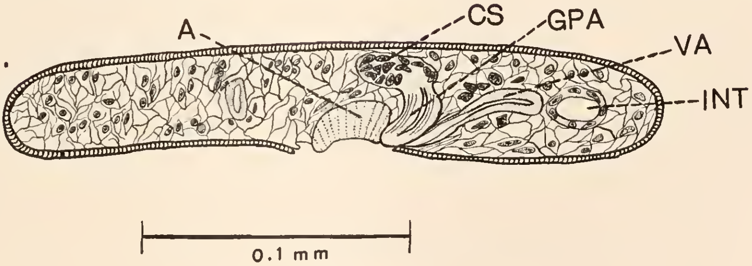
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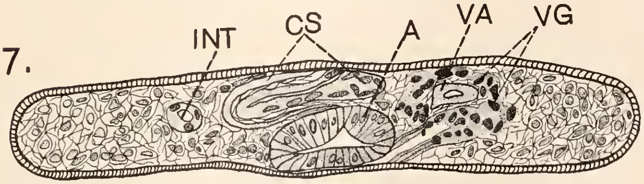
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PLATE II

FIG. 4. Excretory system of an adult *M. arenaria.*
FIG. 5. Mature, encysted metacercaria of *M. arenaria.*
FIG. 6. Transverse section, *M. arenaria,* through region of genital papilla.
FIG. 7. Transverse section, *M. arenaria,* through region of vagina and vaginal glands.

In most respects, as shown in Figs. 1 and 2, the metacercaria and adult are identical. Smaller size and absence of eggs, however, distinguish the metacercaria from the adult.

Marie Lebour (1908, 1911) described a trematode cyst occurring in barnacles of the Northumberland coast. This cyst was described as thin-walled and spherical, measuring 0.04 mm. across, and liberating a "cercaria" 1.0 mm. long. We suspect that 0.4 mm. is the correct figure for the diameter, as it seems unlikely that a metacercaria 1 mm. long could be contained in a cyst measuring only 0.04 mm. across. The cyst described by Miss Lebour is larger than the metacercarial cyst of *M. arenaria,* and *C. balani* Lebour, 1 mm. long, is longer than any metacercaria *or* adult which we have obtained. In *C. balani* Lebour, the prepharynx is very long, longer than the oesophagus, while in the meta-

TABLE I

Measurements of adult and metacercaria of Maritrema arenaria.

	Living adult (under No. 2 cover slip)	Preserved adult stained whole mount (range in 10 specimens)	Living metacercaria (under No. 2 cover slip)
Body length	0.858 mm.	0.303–0.566 mm.	0.576 mm.
Body width (region of testes)	0.380 mm.	0.192–0.266 mm.	0.360 mm.
Acetabulum	0.055 mm.	0.038–0.050 mm.	0.055 mm.
Oral sucker	0.055 mm.	0.038–0.054 mm.	0.055 mm.
Prepharynx	0.034 mm.	0.010–0.014 mm.	0.019 mm.
Pharynx	0.034 mm.	0.020–0.030 mm.	0.028 mm.
Oesophagus	0.153 mm.	0.064–0.10 mm.	0.150 mm.
Intestinal crura	0.239 mm.	0.142–0.208 mm.	0.220 mm.
Left testis	0.087 × 0.112 mm.	0.036 × 0.064–0.076 × 0.088 mm.	0.080 × 0.110 mm.
Right testis	0.087 × 0.112 mm.	0.040 × 0.060–0.070 × 0.10 mm.	0.074 × 0.110 mm.
Ovary	0.068 × 0.146 mm.	0.030 × 0.054–0.062 × 0.084 mm.	0.050 × 0.070 mm.
Egg	0.010 × 0.020 mm.	0.006 × 0.016–0.007 × 0.020 mm.	none

cercaria of *M. arenaria* the prepharynx is markedly shorter than the oesophagus. In the former, the body of the animal is covered with spines even at the posterior end, while in the latter the spines do not extend posterior to the level of the middle of the ovary. Miss Lebour states that the animals were badly preserved, and that measurements of the organs were not made from the living animal. Unfortunately it seems impossible with the amount of information available to state definitely that *Cercaria balani* Lebour and the metacercaria we have found are the same form—or that they are different. Lebour suggested (1908) that *C. balani* might be the larval form of *Spelotrema excellens* Nicoll, but later (1911) retracted this suggestion. Nicoll and Small (1909) found *C. balani* Lebour to bear more resemblance to *Levinseniella brachysoma.* Since *Spelotrema* and *Levinseniella* are also members of the *Microphallidae,* it seems probable that the two metacercariae must be, if not the same form, closely related ones.

Life History of Maritrema arenaria

The metacercariae found in barnacles of the Woods Hole region and the adult fluke found in the gut of the ruddy turnstone, *Arenaria interpres,* resemble each other very closely, the chief difference being a greater development of the reproductive system and a greater body size in the adult. On this basis alone, it would be possible to state that the form encysted in *Balanus* is the metacercaria of *M. arenaria.* Corroboration is furnished by study of the feeding habits of *Arenaria,* and examination of the gut of freshly-killed birds. The turnstone is found most frequently on rocky shores, where it obtains food by turning over small stones with its bill, and by searching the surface of the larger rocks, which are usually covered with barnacles. Observations on the feeding habits of *Arenaria* led us to consider it as a possible adult host for our metacercaria, which we had found before the host of the mature worm was known. The gut of the first bird killed and examined contained bits of barnacle shell, whole and partially digested barnacles, partially and freshly excysted metacercariae, and 10,000–12,000 adult *M. arenaria.* A similar condition was found in a dozen turnstones subsequently investigated. It was impossible to obtain an uninfected bird for experimental excystment of metacercariae, as *Arenaria* breeds within the Arctic circle, and is heavily infected before reaching this region. While we have not been able to secure excystment after experimental feeding of the following animals—young gulls (*Larus argentatus*), young terns (*Sterna hirundo hirundo*), spotted sandpiper (*Tringoides macularius*), white mouse, white rat, kitten, and domestic fowl—we feel certain that the metacercaria from *Balanus* is an immature form of *M. arenaria.*

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Extensive surveys in the Woods Hole region showed the incidence of infection to be very high in *Balanus balanoides,* except for surf-swept shores, where rocks bear only small barnacles. Indeed, so extensive is this infection that uninfected barnacles proved to be a notable exception in one region after another. The number of metacercarial cysts per barnacle, ranging from 1 cyst to upwards of 1,000 cysts, and predominantly averaging between 30 and 100 cysts per individual, indicated either an abundance of cercariae, or retention of the encysted parasites over long periods of time. Studies of a number of authors, including Faust (1932) and Rothschild (1937), make it seem probable that the cercaria is a Xiphidiocercaria of the Ubiquita type (Sewell, 1922), and therefore a minute, feebly-swimming form. With the two above facts in mind, it was natural to expect to find the cercariae of this fluke developing in a molluscan host living in close proximity to barnacle-covered rocks. Mollusks common to such a habitat near Woods Hole are *Littorina littorea, L. rudis, L. palliata, Urosalpinx cinereus, Mitrella lunata, Mytilus edulis, Modiolus demissus, Mya arenaria, Petricola pholadiformis, Anomia simplex,* and *Crepidula fornicata.* Over a period of two summers, hundreds of individuals of each of the above-mentioned mollusks were carefully observed, both in living and crushed condition. Aside from cercariae of known life cycles, but two cercariae were found as possible suspects, *Cercaria parvicaudata* (Stunkard and Shaw, 1931) and a form tentatively identified as *C. ubiquitoides* (Stunkard, 1932). Repeatedly, uninfected or lightly infected *Balanus* individuals were exposed to free cercariae of these two types with completely negative results.

Rees (1936) reports an ubiquitous cercaria found in *Littorina.* In February and March, 20 out of 50 specimens of *L. rudis,* 4 out of 40 specimens of *L. palliata* (*obtusata*), and 2 out of 2,000 specimens of *L. littorea* were infected. The cercaria closely resembles *C. ubiquitoides* Stunkard (1932), and is also similar to *C. ubiquita* Lebour (1907). We have been unable to identify any cercaria found in the Woods Hole region during the summer as the form reported by Rees, or by Lebour. Cable and Hunninen recently described (1938) an ubiquitous cercaria, *C. nassicola,* as yet unconnected with any known life history.

All species of snails common to shores with heavily infected barnacles were repeatedly exposed to the mature eggs of *M. arenaria* for weeks, following which these snails proved to be devoid of cercariae.

In the light of this evidence, it is suggested by the authors that the completion of this life cycle may resolve itself into a careful study of the mechanics or chemistry of the infection process, or a study of the possibilities that the period of infection is limited seasonally. The migration dates of the turnstone, the adult host, are such that fresh infections of the molluscan hosts of this region must fall between May 1 and June 8 (June 24), and between (July 4) July 24 and October 16. Allowing for a period of development of cercariae, and assuming that the period of cercaria production, once begun, may be limited, it appears possible that the cercariae are not present in the Woods Hole region during the summer months.

Summary

A new species of the trematode genus *Maritrema, Maritrema arenaria,* is described as to morphology, adult host (*Arenaria interpres*), and metacercarial host (*Balanus balanoides*). Negative results are reported from a search for the sporocyst and cercarial stages of this fluke, and the suggestion is made that the cercaria may not be present during the summer months in the Woods Hole region.

Acknowledgments

We should like to express our sincere appreciation of the helpful advice and criticism given by Dr. J. S. Rankin, Jr., Amherst College; Dr. W. E. Martin, De Pauw University; and Dr. H. W. Stunkard, New York University. We are also indebted to Dr. Rankin and to Dr. B. R. Coonfield, Brooklyn College, for their help in securing living material for study.

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Vol. LXXVIII, No. 3 June, 1940

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE REPRODUCTIVE CYCLE OF THE VIVIPAROUS TELEOST, NEOTOCA BILINEATA, A MEMBER OF THE FAMILY GOODEIDAE

II. The Cyclic Changes in the Ovarian Soma during Gestation

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Introduction

In an introductory article on the reproductive cycle of *Neotoca bilineata*, an account was given of the breeding cycle of the female as observed in the laboratory (Mendoza, 1939). In this second paper of the series, a description is given of the changes which the ovarian soma undergoes during gestation. Previous articles on the reproductive cycles of viviparous teleosts usually have failed to give complete accounts of the somatic cycle although the later papers on *Fitzroyia lineata* (Scott, 1928), *Xiphophorus helleri* (Bailey, 1933), and *Cymatogaster aggregatus* (Turner, 1938) are notable exceptions. It is the purpose of this account first, to supplement the original, brief description given by Turner in 1933 and second, to contribute to a better understanding of the marked changes which a typical viviparous ovary undergoes during gestation. The discussion includes (1) a brief reference to the gross morphology of the ovary, (2) the general ovary-embryo relations, and (3) a detailed account of the cyclic changes in the ovigerous folds, the epithelium, the free cellular elements, and the blood supply.

Materials

The ovaries used in this investigation are from females whose breeding cycles were closely followed in the laboratory (Mendoza, 1939). A complete series was obtained ranging from virgin and non-gravid ovaries through all stages of gestation to an ovary undergoing involution three hours after the birth of the young (Table I). In the discussion to follow, ovaries will be referred to, not by the number of the female, but by the length in millimeters of the contained young (3.5, 7.2, etc.). Thus the emphasis is placed not on any one individual female but on the conditions typical of that particular stage of gestation. Ovaries earlier

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than 1.5 will be designated appropriately. Most of the larger ovaries were sectioned after the young had been removed from one side and measured; the smaller ovaries were sectioned intact. The fixing fluid used most was that of Bouin although Zenker's, Fleming's, and corrosive sublimate were used for special fixations. The alcohol-xylol series was used almost entirely for dehydrating and clearing. All embedding was done in paraffin and serial sections of each ovary were made at a thickness of 10 micra. Iron hematoxylin was used as the standard stain with orange G as the principal counterstain although eosin Y and acid fuchsin were used occasionally. The stains of Mallory, Van Giesen, and a modified Bielschowsky (Foote and Menard, 1927) were used to differentiate connective tissue whereas Fleming's fixation identified fat and Mayer's muci-carmine was used as the test for mucin. All photomicrographs were taken with a Zeiss microscope and a Reflex-Korelle camera.

TABLE I

The complete series of ovaries sectioned and examined during the study of the somatic cycle of the ovary.

Number of ovary	Stage of embryos	Number of ovary	Stage of embryos
No. 3	Ovocyte (non-gravid female)	No. 14	3.0 mm.
4	“ “ “	44	3.5 “
6	“ “ “	8	4.5 “
15	“ “ “	12	4.5 “
20	“ “ “	23	6.0 “
56	“ “ “	27	6.0 “
74	“ “ “	9	7.0 “
65	blastodisc	11	7.2 “
22	early segmentation	1	9.0 “ (birth of young)
73	“ “		
19	late segmentation	66	3 hours after birth of young
47	1.5 mm.*		
16	2.7 mm.	54A	virgin female
		54B	“ “

* The numbers in millimeters represent the average length of the contained young in one side of the ovary.

GROSS MORPHOLOGY

The ovary is a single structure approximately 5–10 mm. long, colorless, spindle-shaped, and with no external lobulations; it lies in the extreme dorsal portion of the pleuro-peritoneal cavity, attached by a short mesovarium along the median dorsal line of the coelom. As is true of all the viviparous teleost ovaries known to the writer, the gonad and its single oviduct are continuous, with no marked line of demarcation between the two. The structural relations within the ovary are fundamentally similar to those present in other strictly viviparous teleosts, namely, an external sac to which are attached in a diverse manner lobulated folds bearing the germinal tissue. In *Neotoca*, the ovary is divided into two longitudinal chambers by a heavy perforated septum in the median sagittal plane. Each chamber in turn is filled almost completely by a large, lobulated, ovigerous fold attached by a narrow strip of tissue along the dorso-lateral wall of the ovarian sac (Fig. 1). The internal surface of the ovarian wall is characterized in turn by low, longitudinal folds. In the region of the oviduct, the median septum and the ovigerous folds are absent; only the ridges or folds along the wall remain, accentuated in size (Fig. 9).

The tissues involved in the ovary are few. A continuous internal epithelium lines the ovarian cavity completely investing the ovigerous folds, the ovarian wall, and the median septum (Plate I). A loose stroma of connective tissue under the epithelium forms the bulk of the ovigerous folds (Figs. 1–6, 11, 12), the septum, and is continued around the ovarian wall as a narrow layer. Forming the external wall of the ovary is a heavy layer of circular muscle richly interspersed with collagenous connective tissue fibers (Figs. 1, 21); the muscle layer in turn is invested by a serosa. Four pairs of large blood vessels surrounded by very heavy coats of muscle and connective tissue course along the wall of the ovary at the ends of the median septum and at the base of each ovigerous fold (Fig. 1). Smaller branches ramify to a rich, subepithelial plexus of capillaries (Fig. 10). A complete description of the germ cells which occur within the stroma of the folds will be reserved for a later paper.

Embryo-Ovary Relations

The ovocytes develop within follicles in the ovigerous folds (Fig. 1), are fertilized while still within the follicle, and are expelled immediately into the ovarian lumen (Fig. 2). Consequently, the entire developmnt of the embryos occurs within the lumen of the gonad proper, within the limited space left between the ovigerous folds and the ovarian wall. Although no special relations are established between the embryo and the ovary as in *F. lineata* where processes of the ovarian tissue actually penetrate into the branchial chamber of the embryos, in *Neotoca* the young always remain in close proximity to the ovarian folds. During gestation, the ovary undergoes radical modifications which again subside with birth of the embryos. Accompanying these changes, the young likewise become modified, developing huge absorptive processes that are resorbed at time of birth (Turner, 1933, 1937; Mendoza, 1937). In view of the scarcity of yolk in the ova, it has been assumed that the marked modifications on the part of the ovary and the embryo are to supply the necessary nutritional and respiratory relations between the two. It is, therefore, with the cyclic somatic changes undergone by the ovary during gestation that the present paper is concerned.

Cyclic Changes

The most conspicuous changes in the ovary are: (1) an increase in the dimensions of the ovary, (2) a swelling of the ovigerous folds, (3) a marked secretory activation of the internal epithelium, (4) an increase in certain wandering cells of a secretory nature, and (5) an increase in the vascularity of the ovary.

Size

Although a resting ovary averages approximately 1.5 mm. in diameter (Fig. 1), a gravid ovary at term measures 10–15 mm. in diameter (Fig. 7), occupies the greater part of the coelom and distends the abdominal

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EXPLANATION TO FIGURES

All figures are photomicrographs taken with a Zeiss microscope and a Reflex-Korelle camera.

Plate I

A series of cross-sections of ovaries, photographed under the same magnification and showing the gross changes of the ovary and young during gestation. This series is completed with Figs. 7–9 on Plate II. The scale of magnification for this series appears in Fig. 9 of Plate II.

Fig. I. A non-gravid ovary. Clearly visible are: the outer muscular wall, the internal epithelium of the ovary, the median septum, one highly lobulated ovigerous fold on each side of the median septum, the paired blood vessels appearing especially distinct at the base of each of the ovigerous folds, and developing oocytes which appear as round structures within the ovigerous folds. The cut in the wall is the incision normally made in most ovaries before fixation.

Fig. 2. An ovary with young in stages of germ-layer formation. One normal embryo appears just below the center of the ovary. The folds are slightly swollen.

Fig. 3. An ovary with young 1.5 mm. in length. The folds are swollen and the epithelium is starting to secrete. A section through the tail region of one embryo appears in the upper right.

Fig. 4. An ovary with young 3.5 mm. in length. The folds are swollen and the epithelium is at its maximal point of secretory activity. One young occupies almost the entire left side of the figure.

Fig. 5. One-half of an ovary with young 4.5 mm. in length. The ovarian wall is more tenuous because of the growth of the embryos. Two young fill one-half of the ovarian lumen.

Fig. 6. A small section only of an ovary with young 6.0 mm. in length. The ovigerous folds are still swollen; two young are visible.

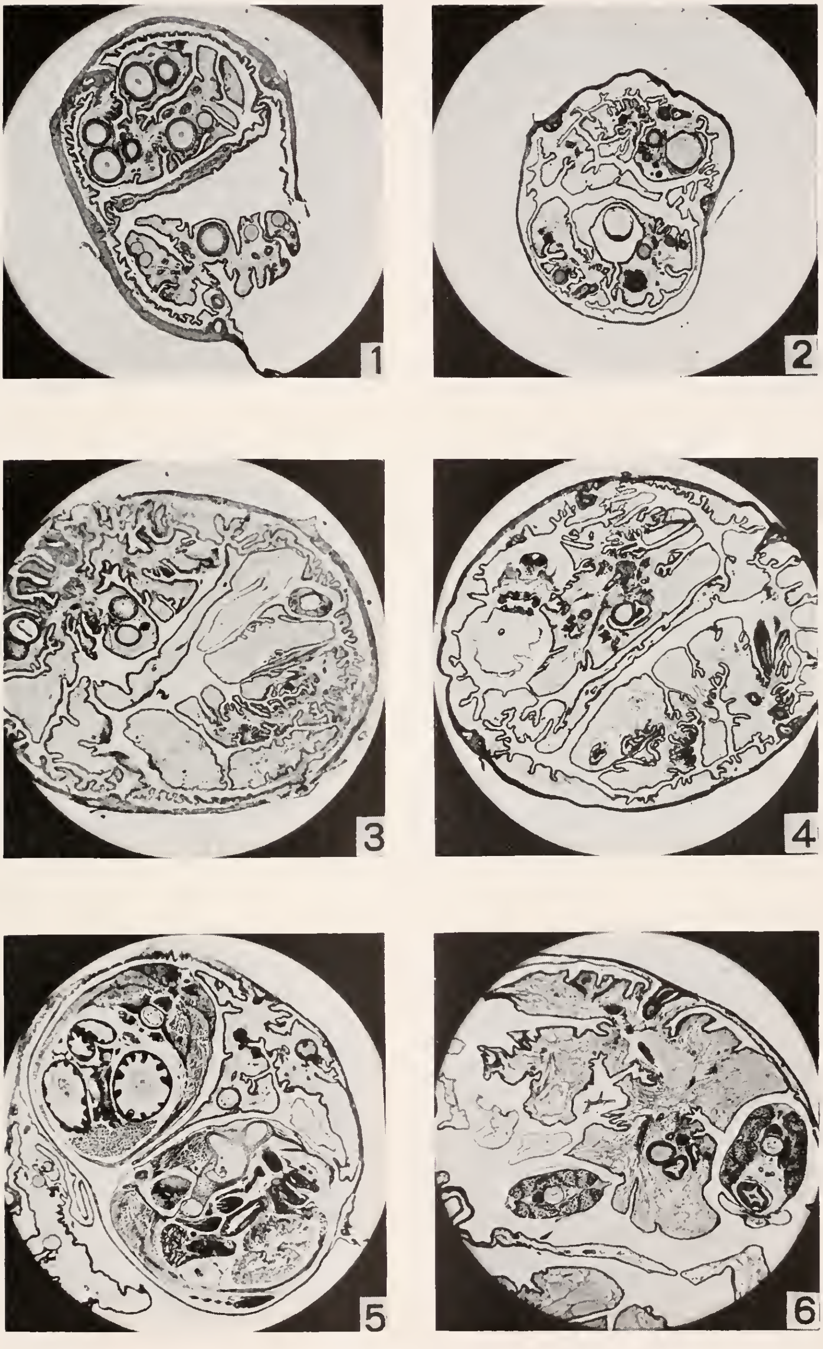

PLATE I

wall of the female far beyond normal proportions. Simultaneously, due to extreme expansion, there is a resulting decrease in the thickness of the muscular wall from an average thickness of 55 micra in a resting ovary (Fig. 21) to 5–10 micra in an ovary just prior to the birth of the young (Fig. 22), a decrease to almost one-tenth of the original thickness.

Ovigerous Folds

The ovigerous folds consist of a loose connective tissue reticulum completely invested by the internal epithelium of the ovary. Within the folds but adjacent to the surface lie the nests of developing ovocytes (Fig. 1). In the resting ovary, the fibers of the reticulum are loose, wavy, and branched (Fig. 11). The greater part of the reticulum is composed of thin delicate fibers; however, there occur occasional masses of heavy fibers scattered at random in the stroma and especially underneath the epithelium. Differential staining shows that the delicate fibers are of a collagenous nature whereas the heavy fibers not only are collagenous but in addition show a marked affinity for argyrophylic or reticular connective tissue stains. In the interstices of this tissue there occur scattered droplets of an oily nature and small quantities of a lymph-like fluid that coagulates upon fixation. In addition to the fluid bathing the reticulum there also occur many types of wandering cells to be described later. Although in the typical resting ovary the ovigerous folds are small, compact, and with but little contained fluid (Fig. 1), advance in gestation is accompanied by marked changes. The folds begin to

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PLATE II

FIG. 7. A very small portion of an ovary with young 9.0 mm. in length and ready for birth. The ovigerous fold is collapsed and appears in the upper center of the figure. The two delicate structures in the center between the large embryos are sections of the absorptive processes of the young. The large black spots in these and other absorptive processes in the figure are blood vessels that supply the superficial capillary plexus of the processes.

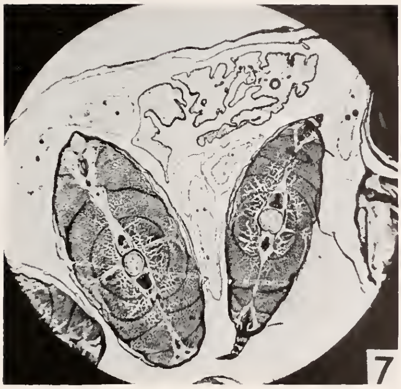
FIG. 8. An ovary three hours after the birth of young showing the nearly complete involution of the gonad. These and all preceding figures were taken under the same magnification and give an idea of the radical changes in size the ovary undergoes during gestation.

FIG. 9. A section of the oviduct. The scale represents the final magnification for all figures from 1–9.

FIG. 10. An oil-immersion photograph showing the subepithelial capillary plexus in an ovary during the latter stages of gestation (7.0 mm). The black rings that show prominently in the lower section of epithelium are heavy connective tissue fibers that surround the capillaries. Some erythrocytes are visible within the vessels.

FIG. 11. A high-power view of a portion of an ovigerous fold in a non-gravid ovary.

FIG. 12. A high-power view of a portion of an ovigerous fold in a gravid ovary. The fold is swollen by the contained fluid. The reticulum of the stroma is stretched like a taut network. The scale for this and the preceding figure appears at the lower left.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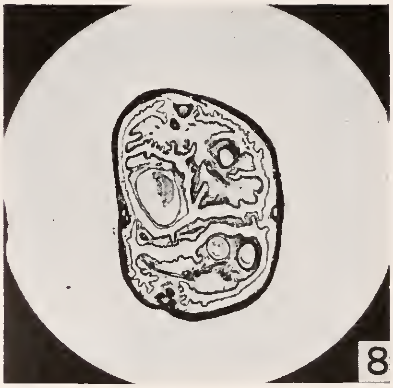
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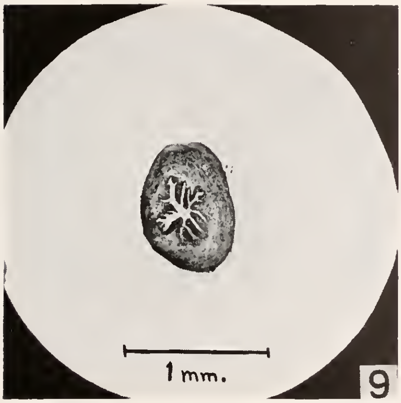

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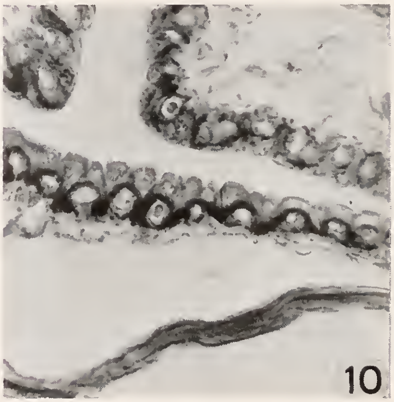
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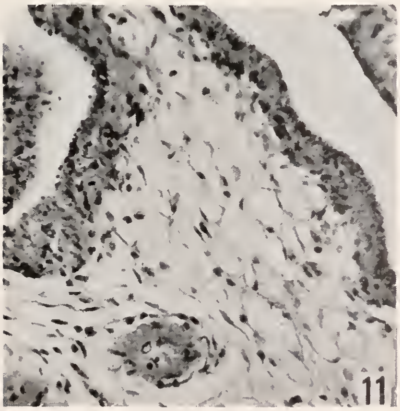
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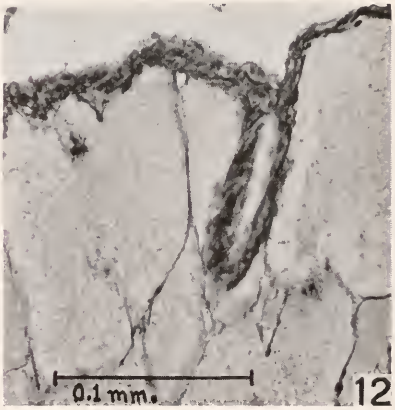

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PLATE II

swell noticeably with a resulting turgidity caused by the increase of the contained fluid (Fig. 2); these changes become prominent when embryos reach stages of segmentation and germ layer formation. The maximal condition of swelling and turgidity of folds is reached between stages 1.5 and 6.0 at which time the folds usually are large, rounded, and seemingly full of a flocculent substance (Figs. 3, 4, 6). The pressure of the fluid may be so great at times that individual cells or an entire section of epithelium may become elevated from the surface of the folds. In addition, the reticulum usually becomes stretched like a taut net within the folds (Fig. 12). It is evident, consequently, that the increase in the size of the ovary during gestation is caused not only by growth of the embryos but also by the marked swelling of the ovarian tissues. Finally, in the last stages of gestation, the folds which are no longer rounded often become moulded in their contour by the presence of the large embryos; the folds actually have the appearance of being depleted or collapsed, their purpose apparently having been served. There is little or no coagulated fluid and but few wandering cells so prominent at other times (Fig. 7). Three hours after birth of the embryos (Fig. 8), the folds appear to all purposes like those in a normal non-gravid ovary. Although there is some variation in the time of appearance and disappearance of the swollen condition, the typical cycle is as explained above.

It will be recalled that this reticulum of connective tissue is continued as a narrow layer around the non-ovigerous wall of the ovary, between

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PLATE III

A series of photomicrographs showing the changes in the ovarian epithelium during gestation. The series is completed with Figs. 19 and 20 on Plate IV. All photographs are magnified equally, approximately 620 ×.

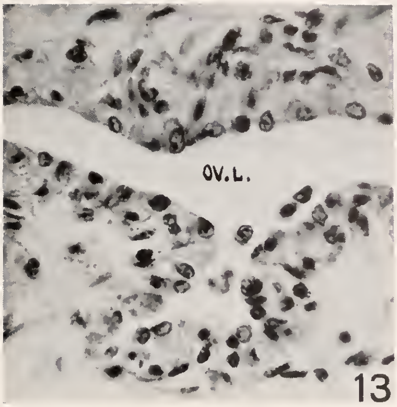
FIG. 13. A non-gravid ovary in which the epithelium is represented by cells with small, prominent nuclei and with little cytoplasm. Nuclei appear to intermingle with underlying fibrocytes. The ovarian lumen (*OV.L*) appears in the center.

FIG. 14. An ovary with embryos in late segmentation stages. The epithelium (*EP*) is better defined than before since the cytoplasm is far more conspicuous. The cells are preparing for secretory activity.

FIG. 15. An ovary with young 2.7 mm. in length. The cells of the epithelium are tremendously enlarged and actively secreting.

FIGS. 16 AND 17. Two views of the same ovary during maximal secretory activity. The young are 3.5 mm. in length. In both figures adjacent epithelia have become temporarily adhered to each other.

FIG. 18. A section of the median septum in an ovary with young 4.5 mm. in length. The epithelium appears to be decreasing in secretory activity; the cytoplasmic volume of the cells not only appears to be reduced but frequently there is a partial or complete collapse of the cell.



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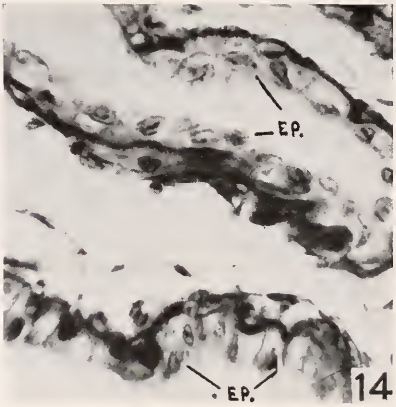

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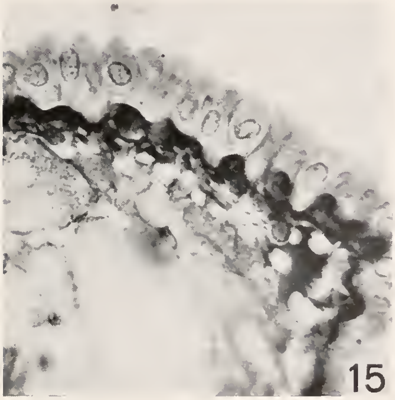

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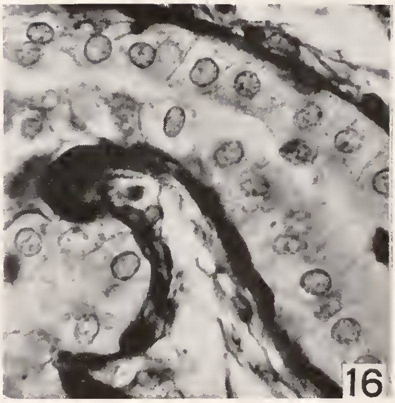

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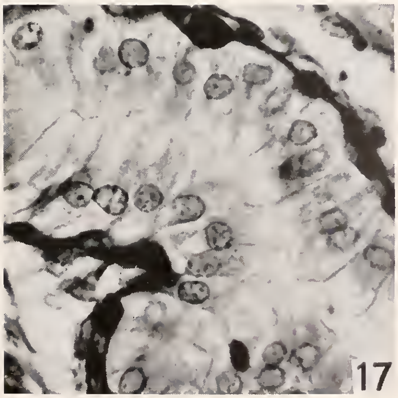

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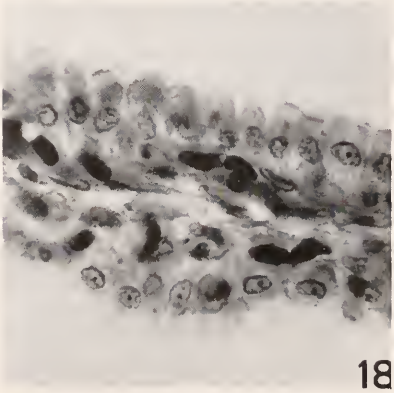

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PLATE III

the internal epithelium and the outer muscular wall, and into the median septum. These regions, continuous with the stroma of the ovigerous folds, suffer identical changes (Figs. 3, 4). The increase in quantity of fluid with its resultant turgidity likewise affects these parts, all of them contributing to the spongy nature of the ovary during gestation.

Epithelium

The epithelium forming the inner surface is subject to as great a change as are the actual folds. In most resting ovaries, the epithelial cells have very prominent darkly-staining nuclei but with indefinite cell walls and very little cytoplasm (Fig. 13). The epithelium is flattened, almost embedded, against the subepithelial network of heavy connective tissue fibers identical with the similar ones in the ovigerous folds. This disorganized appearance usually is lost completely in stages of early segmentation for, whereas the cells may not be large, they become cuboidal in shape and regularly arranged at the surface (Fig. 14). During later stages, the cells of the internal epithelium of the ovary become columnar and greatly swollen (Fig. 15); they actually fuse with cells on adjacent ridges wherever they come into contact (Fig. 16). The cytoplasm is prominent now and well-defined having the typical appearance of that of a secretory cell. This enlarged condition of the epithelial cells, especially along the ovarian wall, is very characteristic of these and later periods of gestation. Between stages of 1.5 and 3.5, secretory activity reaches a maximum, simultaneous with the greatest turgidity of the ovigerous folds (Fig. 17). Whereas cell identification in resting ovaries is difficult (Fig. 13), during these later stages each cell stands out clearly, is tremendously enlarged, actively secreting, and frequently is disrupted at its distal end. The marked similarity between this epithelium and that of a mammalian uterus during the height of secretion is truly remarkable. This secretory activity continues later on though in a more abated fashion. On the whole, the cells now are more disorganized, collapsed, and some even are being sloughed off into the ovarian lumen; the general impression given is that of the abatement or cessation of secretory activity. This spent appearance reaches a maximum during late gestation (stages 6.0 and 7.2) when the cells show shrinkage and separation from adjoining cells (Fig. 10). In the last phase of gestation (9.0) the epithelium once more regresses to a thin cellular layer, giving no indication of secretory activity and flattened against the underlying subepithelial capillary plexus (Fig. 19). This condition is particularly true for the epithelium along the wall which has been extended so greatly that fibrocytes of the subepithelial connective tissue, capillaries with their contained erythrocytes, and the epithelial cells themselves, to all appearances, form a continuous layer (Fig. 22). Finally, three hours after the birth of the young when the involution of the ovary is nearly complete, the epithelium again changes; it shows a definite attempt to readjust itself after the radical extension undergone during gestation. The cells now are small, with prominent, vesicular nuclei, devoid of practically all cytoplasm, and very loosely attached (Fig. 20). Many cells actually are superimposed; many others are being lost into the ovarian lumen. It is interesting to note that the large quantities of secretion elaborated during the early and middle stages of gestation now are completely lost. There is nothing left but the mere skeleton of the formerly enlarged cells which now apparently are in a state of physiological exhaustion.

This description of changes in the internal epithelium is characteristic of its entire extent, not only the investment of the ovigerous folds and of the median septum, but particularly of the ovarian wall.

Free Cellular Elements

In addition to the changes of the primary or permanent elements of the epithelium, there are equally important changes in the free cells or secondary elements that not only wander throughout the extent of the submucosa-like reticulum but also penetrate into the epithelium. It is evident that these cells contribute greatly to the swollen condition of the gravid ovary. The first cell to be described hereafter is called simply a secretory cell. It appears in almost all stages of early gestation, being present in the non-gravid ovary in variable numbers and increasing in number until the height of secretory activity in the epithelium. Then, the cell undergoes a marked transformation and decreases in number from stage 4.5 until the end of gestation. In stage 9.0 and in an ovary three hours after the birth of young, only few such cells occur. The tremendous increase in the number of these cells cannot be appreciated without numerical evidence. In earlier stages, 15–35 cells per section represent an average number but, in stage 3.5 (Fig. 24) an actual count of 798 cells in one typical section was made. The transformations undergone at this time are unique. An intracellular reticulum, attached to the a-centric nucleus, swells to a bursting point; it then collapses but the cell membrane remains intact with the contained fluid. The cell wall eventually ruptures and collapses, discharging the contents into the surrounding fluid. Since these cells wander throughout the ovary but eventually migrate into the epithelium, the elaborated contents are discharged mostly into the ovarian lumen. The identity and origin of the cell still are uncertain; nothing at all similar has been found in the literature although absence of the cell from the blood stream precludes classifying it as a vascular element. With but few exceptions, its marked increase in numbers up to the time of maximal epithelial secretion and its radical transformation at that time leave little doubt of its contribution to the physiological state of the ovary. It is likely though not yet proven that the secretion elaborated by these cells may be of a nutrient nature.

A second cell type of importance is a granular cell of a variable nature. The nucleus is a-centric and picnotic; the large granules in the cytoplasm may be all basic, all acidophylic, or of a mixed character. The cell is amoeboid in nature and occurs throughout the ovary (Fig. 23). The conclusion that the cell is strictly a tissue granulocyte is drawn because of its complete absence from the blood stream. In resting ovaries it is present although the number varies somewhat; however, as the ovigerous folds approach maximal turgidity, these granular cells appear in maximal numbers. With the decrease of the secretory activity of the epithelium and the turgidity of the folds, these cells likewise diminish. In the latter stages of gestation, very few if

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Plate IV

Fig. 19. The internal epithelium in the last stage of gestation preceding the birth of the young (9.0 mm.). The most prominent structure at the surface is the capillary plexus appearing as a series of rings. Stretched over the plexus appear three cells of the epithelium (arrows). Only the nuclei of the epithelium are prominent; the cytoplasmic content is negligible. A similar stage in which the capillary plexus appears more distinctly is represented in Fig. 10.

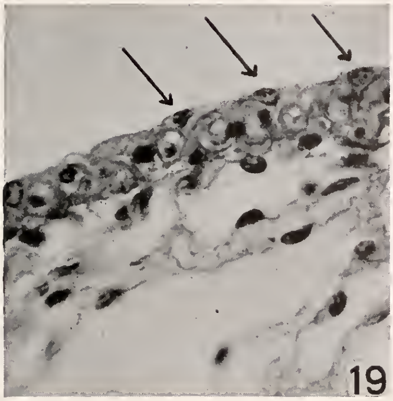
Fig. 20. The epithelium in an ovary three hours after the birth of the young. With the release of the tension on the ovary, the epithelium again becomes well-defined but the vast quantities of secretion and the cytoplasm have been discharged during gestation; only nuclei with a prominent chromatin mass remain. The cytoplasm forms a delicate sheath around the vesicular nucleus.

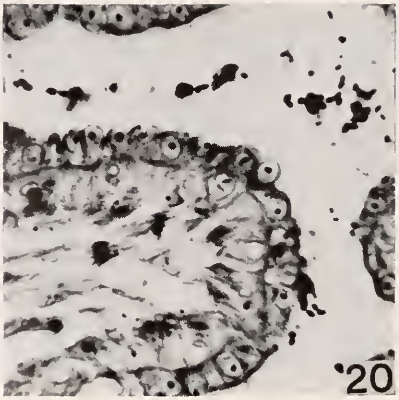
Fig. 21. The ovarian wall in a non-gravid ovary. The internal epithelium is at the upper edge of the figure; the muscular wall is at the lower edge. Although the nuclei of the muscle fibers are plainly visible, the collagenous connective tissue fibers do not show well.

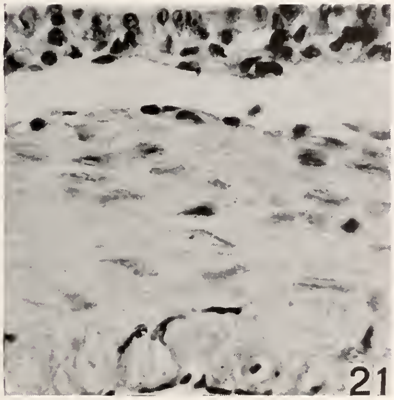
Fig. 22. The ovarian wall just before the birth of young. The magnification is the same as for the preceding figure. The tenuous epithelium again is at the top; the muscular wall is at the bottom. In the epithelium, three cells (arrows), few erythrocytes, and few fibrocytes can be distinguished. This epithelium appears as if under more tension than in Fig. 19 because in the preceding figure the epithelium is from the ovigerous folds, here, the epithelium is from the ovarian wall. The scale represents the magnification in Fig. 10 and all figures on Plates III and IV.

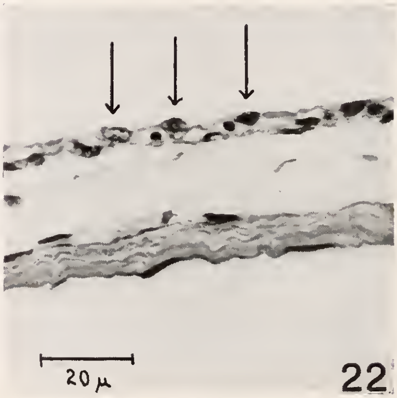
Fig. 23. A granular cell. The nucleus and some of the granules are in focus.

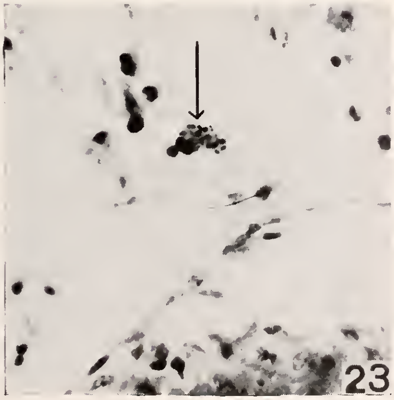
Fig. 24. Wandering secretory cells in the epithelium of an ovary actively secreting (stage 3.5 mm.). The darker bodies in the epithelium are secretory cells partially out of focus. The secretory cell identified by an arrow shows the details of the cell membrane, the picnotic nucleus at one end of the cell, and the intracellular reticulum attached to the nucleus.











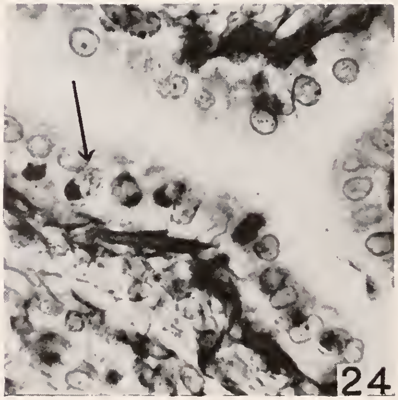


PLATE IV

any of these cells can be distinguished. Further evidence that these cells occur chiefly during the swollen condition of the ovary lies in the presence of the cells in large numbers in two virgin ovaries, ovaries that were in a very swollen condition. The significance of these activated virgin ovaries will be considered later. Whereas the secretory cells penetrate into the epithelium in large numbers, these cells seldom have been seen in the epithelium proper. That these cells accompany or contribute to the spongy condition of the ovary is verified further by actual disintegrating cells and the discharge of their contents into the surrounding medium. The secretion phenomenon through the solution of the huge granules is not improbable since a similar phenomenon has been reported before for eosinophilic cells (Jordan and Speidel, 1924).

Other cells of a primitive nature identified as stem cells from which are derived several of the free cellular elements also occur in large numbers. However, they follow the same cyclic behavior as followed by the two cells described above. Evidently the stem cells contribute to the swollen condition of the ovary only indirectly in so far as they give rise to other active secretory cells.

Blood Supply

Mention has been made already of the subepithelial vascular plexus. During resting phases, the epithelial blood supply is but little in evidence whereas in many ovaries there is a very rich blood supply to the maturing follicles. However, with the progressive development of the embryos, the general circulation increases and emphasis is placed, not on the follicular circulation but on the very prominent subepithelial plexus which remains most conspicuous throughout the rest of gestation (Fig. 10). It is likely that one of the most important rôles of this plexus is concerned with the respiratory gas interchange of the ovarian fluid.

Discussion

There can be no doubt that the ovary of *Neotoca* undergoes very definite cyclic modifications during gestation. The problem is whether this somatic cycle arises solely in response to the presence of the embryos or whether the cycle is inherent, appearing at stated intervals in the female. In other words, the problem is whether or not the ovary undergoes a periodic activation similar to that of the mammalian uterus. The problem arose when it was noticed that one or two of the resting ovaries differed from others in showing some evidence of swelling of the ovigerous folds and an increase in the secretory cells. Two known virgin females then were killed for examination. Far from being in a resting condition, the ovaries were highly swollen, contained large numbers of granular cells, a typical resting epithelium, and but few secretory cells. This evidence further confirmed the presence of the granular cells only at a time simultaneous with the turgidity of the folds. Furthermore, these and other ovaries showed that the secretory cells may appear in variable numbers during early or resting stages but that the complete activation of the cells occurs only during the height of epithelial secretion. Lastly, the ovaries showed that they undergo partial activation even in the virgin condition without the inciting presence of the male or of embryos. In the first article of the series (1939) the writer showed the probable correlation of this swollen condition with the coloration of the female. At that time it was shown further that the coloration apparently was a cyclic phenomenon appearing at somewhat regular intervals. Hence the inference follows that, evidently, the ovary undergoes this somatic activation periodically. This activation may not be very precise in its rhythmic appearance; indeed, it would be very surprising if it were for it is likely that, judged from an evolutionary consideration, the assumption of the completely viviparous condition by this group of teleosts is quite recent.

The spongy condition of the gravid ovary is not entirely unique for this species since large " lymph spaces " in the ovarian tissues have been reported before for other forms: *Zoarces viviparus* (Stuhlmann, 1887); *Stygicola dentata* and *Lucifuga subterraneous* (Lane, 1903); and *Cymatogaster aggregatus* (Eigenmann, 1892; Turner, 1938). This spongy condition no doubt acts in a buffering capacity similar to that of an amniotic fluid; however, it is evident that it has even more vital functions, probably those of nutrition and respiration. Since the earlier studies of teleost viviparity, it has been suggested and assumed that the ovarian fluid must serve the embryos in just such a vital capacity. Actually, this assumption is not the result of chemical analysis but is largely a logical one based on the necessity for such a phenomenon in addition to the following observed facts: (1) there is an inadequate supply of yolk in the ovum (goodeids and others) to satisfy the needs of the growing embryos; (2) viviparous embryos generally develop vascular structures ideally suited for the absorption and interchange of substances (Turner, 1933, 1937); (3) there is an elaboration of vast quantities of secretion; and lastly, (4) there is the flooding of the ovary with a lymph-like fluid that coagulated upon fixation. Actually, it is likely that the only serious attempt to analyze the ovarian fluid in a viviparous ovary was made by Blake in 1867. He concluded the probable presence of an albumin compound with fat, phosphates, and iron. It is desirable that a thorough chemical analysis of such an ovarian fluid be made for a viviparous teleost so that the assumptions made to date can be confirmed or rejected.

Lastly, the writer wishes to stress the marked parallel that exists between a viviparous teleost such as *Neotoca* and a placental mammal. In both forms there is the retention by the female of the developing embryos. In both forms the embryos are dependent on the female for the necessary nutritional and respiratory media because of the more or less complete absence of yolk in the ova and the retention of the developing embryos. In both forms there is a certain degree of similarity in the organ that houses the developing embryos, the uterus in one case and the ovary in the other. Structurally, both organs are similar in that both possess a highly secretory epithelium, a loose underlying connective tissue followed in turn by a heavy muscular coat and by a serosa. Both the uterus and the ovary of the respective forms undergo cyclic somatic modifications during periods of gestation. Both structures become highly vascular. Secretory activation and tumescence of the tissues occurs in both forms. The appearance of the decidual cells of mammals may have its counterpart in the occurrence of the secretory and granular cells in *Neotoca*. In both forms, highly vascular absorptive structures are developed by the embryos for the necessary nutritional and respiratory interchange; in one case, part of the intimate placenta is formed, and in the other, the embryos develop huge trophotaeniae (Turner, 1937) but remain free of the maternal tissues. And lastly, as was suggested in the previous article, there is the possibility that a period may occur in *Neotoca* similar to that of estrus in mammals. In the same way, it is possible that the activation of the ovary in *Neotoca* is a periodic, inherent one, similar to that of mammals. It is indeed unique that two forms, taxonomically as separated as are the teleosts and mammals, should become adapted in such a similar manner in response to a similar condition.

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THE RELATION BETWEEN OXYGEN CONSUMPTION AND RATE OF REGENERATION [1]

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Previous work (Barth, 1937, 1938*a*) showed that regeneration in *Tubularia* could be inhibited by placing the cut end of the stem in a glass capillary. A lowered O_2 tension was thought to be the cause of the inhibition and experiments in which the oxygen tension was varied showed that the rate of regeneration was closely dependent on the availability of oxygen. This relationship was also noted by Miller (1937), who found that the hydranth always appeared at the end where more oxygen was made available either by a higher oxygen tension or by circulating the sea water.

Further experiments by Zwilling (1939), in which the perisarc was removed from the middle of ligatured stems, showed that regeneration occurred on both sides of the perisarcal opening. This phenomenon could be interpreted to mean that the perisarc does not permit enough oxygen to reach the tissues to enable them to form a hydranth. The removal of the perisarc allows direct access to the oxygen of the sea water and starts the process of regeneration. If the foregoing experiments are to be explained on the basis of the concentration of oxygen at the tissues, then a study of the amount of oxygen consumed by the tissues is necessary. Some observations have been made by Child and Hyman (1926) on *Corymorpha* and Hyman (1926) on *Tubularia*. They found that the distal parts of stems consumed more oxygen than the proximal parts and further that young stems respired at a higher rate than old ones.

The experiments in this paper were designed to test whether: (1) the rate of regeneration of different levels of the stem is proportional to the rate of oxygen consumption at these same levels; (2) the regenerating portion of the stem uses more oxygen than the resting stem; and (3) whether the rate of regeneration of the stem is changed when the rate of oxygen consumption of a stem is increased or decreased.

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[1] These researches were aided by a grant from the Rockefeller Foundation.

Methods

The stems of *Tubularia* were prepared from freshly collected material by cutting them off from the base of the colonies and selecting straight, unbranched stems of uniform thickness and healthy appearance. For the most part the more distal regions of the stem were used as these are free of parasitic growths which introduce an error in measurements of O_2 consumption. They were cut the desired length, care being taken to discard the region just adjacent to the hydranth as this region may exhibit a low rate of regeneration. The stems were then cut into halves, thirds, etc., according to the requirements of the experiment. The rate of regeneration was measured by taking the length of the primordium of the hydranth, the diameter of the primordium and the time at which the primordium becomes separated from the stem by a constriction. The rate of regeneration is then calculated as the volume of the primordium in μ^3 divided by the time in hours (Barth, 1938*b*). The units for rate of regeneration are $\mu^3/\text{hours} \cdot 10^5$.

The O_2 consumption was measured with Warburg manometers and the O_2 uptake calculated as the number of cubic millimeters of O_2 per hour per 10 mg. of dry weight. Weighings were made on a microbalance to 0.001 mg. In some of the earlier experiments the stems were merely selected of the same size and the oxygen uptake calculated for the mass of stems without weighing.

The O_2 Consumption of Parts of Stems

In these experiments the stems were cut into 2, 3, 4, or 5 parts and the rate of O_2 consumption determined. In some cases the rate of regeneration of the hydranth was also measured although previous experiments (Barth, 1938*b*) show that the rate falls off from distal to proximal region of the stem. The results have been calculated on the basis of mm.^{3} O_2 used per hour per 10 mg. dry weight of the stem. Table I gives the results of using distal and proximal halves of stems. Since each half forms a hydranth at both cut ends, the rate of regeneration of the distal (oral, apical) hydranth and proximal (aboral, basal) hydranth of each half is measured. The table shows that the distal half consumes more oxygen than the proximal half (18.7 compared with 11.9) and the rate of regeneration of the distal half is greater (53.2 compared with 34.2). Likewise, in thirds of stems the rates of O_2 consumption and rates of regeneration are highest in the distal third, lower in the middle third and lowest in the proximal.

Table II records the rates of O_2 consumption of fourths of a stem.

Table III shows that the proximal fifth may consume as much O_2 as the third fifth of the stem, although since the stems were not weighed

TABLE I

Rate of oxygen consumption of parts of stems of *Tubularia*.
Rate = mm.^{3} O$_2$/hr./10 mg. dry weight.

Description of Stems			Oxygen Consumption					Rate of Regeneration μ^3/hrs. 10^5	
No.	Length	Region	Temp.	O$_2$	Time	Dry weight	Rate	Distal hydranth	Proximal hydranth
	mm.		°*C.*	*mm.*^{3}	*hours*	*mg.*			
20	7.5	distal half	19 ±.02	89.5	9.54	5.04	18.7	53.2	12.7
20	7.5	proximal half	19 ±.02	60.5	9.54	5.35	11.9	34.2	19.3
15	8	distal half	18.5±.02	112	13.42	3.512	23.8		
15	8	proximal half	18.5±.02	91	13.42	3.645	18.6		
20	5–6	distal third	19 ±.02	46.5	6.0	2.81	27.6	49.0	24.4
20	5–6	middle third	19 ±.02	41.0	6.0	3.06	22.4	34.4	17.4
20	5–6	proximal third	19 ±.02	31.4	6.0	3.36	15.6	23.6	9.6
10	5	distal third	18.5±.02	124	24				
10	5	middle third	18.5±.02	97	24				
10	5	proximal third	18.5±.02	89	24				

the results are not conclusive. This latter result agrees, however, with the experiments of Child and Hyman (1926), who found that the proximal third might sometimes use as much O$_2$ as the middle third. This observation correlates with the earlier observation of Child (1907) that the extreme proximal end sometimes regenerates as fast as higher levels of the stem.

It is clear then that there are regional differences in rate of O$_2$ consumption of the stem after cutting and that the rate of regeneration of the hydranth is roughly proportional to the rate of O$_2$ consumption.

TABLE II

Rate of oxygen consumption of parts of stems of *Tubularia*. In these experiments the proximal pieces were cut a little longer than the distal ones in an attempt to compensate for differences in diameter. As seen from the weight measurements they were cut a little too long and consequently are a little heavy. Rate = mm.^{3} O$_2$/hr./10 mg. dry weight.

No.	Length	Region	O$_2$	Time	Dry weight	Rate
	mm.		*mm.*^{3}	*hours*	*mg.*	
12	3	distal fourth	39.2	16.83	.977	23.8
12	3	second fourth	32.3		1.086	17.7
12	3	third fourth	30.0		1.190	15.0
12	3	proximal fourth	31.8		1.357	13.9
20	3–4	distal fourth	27.0	8.92	1.281	23.6
20	3–4	second fourth	22.8		1.517	17.0
20	3–4	third fourth	14.6		1.614	10.2
20	3–4	proximal fourth	21.2		2.023	11.7

Changes in the Rate of O_2 Consumption during Regeneration

When the values for O_2 consumption are plotted against time as in Fig. 1 the curve is S-shaped, indicating that as the regeneration process proceeds it requires O_2 at an increasing rate reaching a maximum and then falling off in the later stages of regeneration. The rate is highest from about seven to sixteen hours and it is during this period that the size of the primordium is determined (Peebles, 1931). These changes in rate are observed only in the case of short (2–4 mm.) stems.

The S-shape of the curve is lost or almost lost when the data from long (8–15 mm.) stems are plotted (Fig. 2). Here we find that the rate of O_2 consumption is almost constant throughout the period of regeneration. The interpretation given is that the O_2 consumption of the resting stem is so high that, in long stems where the regenerant comprises only about 10 per cent of the stem, the changes in rate caused by the regenerant are not noticeable. On the other hand in short stems, where 50 per cent of the stem may be regenerating tissue, the changes in rate during regeneration are more easily detected.

TABLE III

Oxygen consumption of fifths of stems of *Tubularia*. Figures are for total amount of oxygen in mm.^{3} O_2 consumed at time indicated.

No.	Length	Region	7 hours	26 hours	37 hours
15	2–3 mm.	distal fifth	31.4	125	162
		second fifth	23.0	104	140
		third fifth	18.3	95	125
		fourth fifth	16.8	83	108
		proximal fifth	22.0	94	122
			6.16 hours	17.33 hours	24.75 hours
17	2–3 mm.	distal fifth	17.3	52.0	63.0
		second fifth	14.6	41.5	51.0
		third fifth	12.4	38.9	49.5
		fourth fifth	13.3	36.6	48.5
		proximal fifth	11.8	34.4	46.0

The Rate of O_2 Consumption and Rate of Regeneration at Varying O_2 Tension

The experiments of Miller (1937) and Barth (1937, 1938) indicated clearly that the rate of regeneration depended on the oxygen tension. In the following experiments the O_2 uptake was determined by placing the stems in different gas mixtures in the manometer vessels for most of the period required for regeneration. Then the stems were removed and the rate of regeneration measured. Table IV gives the results. The rate of oxygen consumption falls from 5.4 in O_2 to 1.6 in N_2, while the rate of regeneration decreases from 177 to 0. It is clear that as the O_2 supply is reduced the O_2 consumption of the stem falls off and the rate of regeneration is decreased.

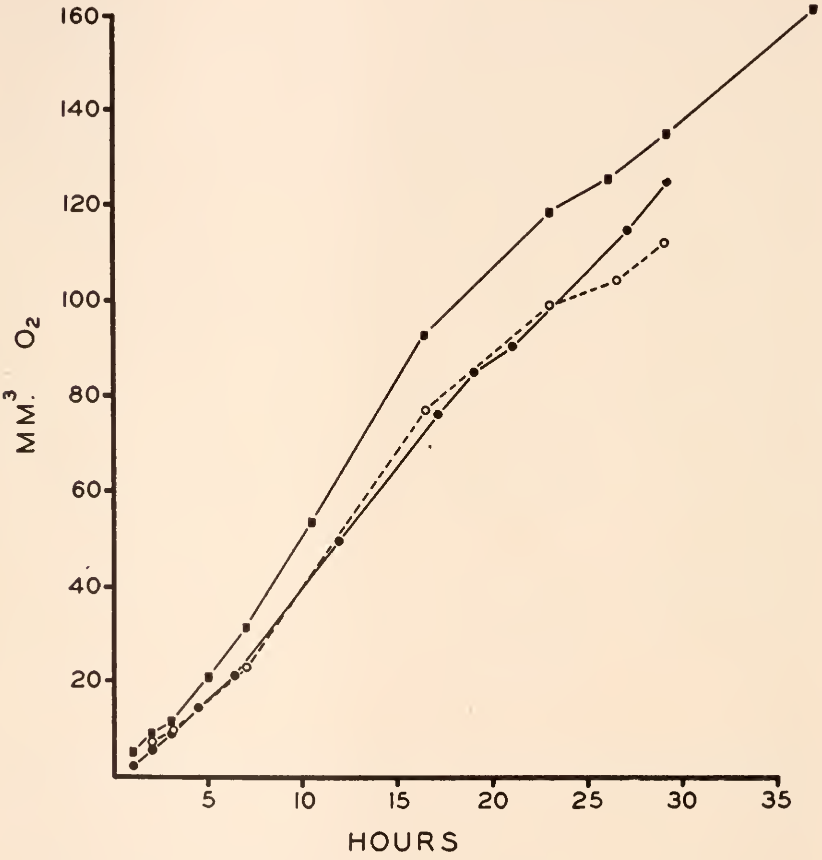

FIG. 1. Total amount of oxygen consumed by short stems, plotted against time. Squares give data from distal pieces of stems 2–3 mm. in length. Open circles are for more proximal pieces 2–3 mm. in length. Solid circles are for distal pieces 4 mm. in length. Hydranths are fully formed at 30 hours.

That the process of regeneration is closely dependent on the O_2 supply is shown by a comparison of the behavior of stems in the Warburg manometers and in open dishes. While stems never regenerate hydranths when ligatured at both ends and kept in open dishes, as many as 50 per cent of stems will regenerate hydranths if ligatured and shaken in the manometers with air. Thus, by keeping a high O_2 tension at the surface of the perisarc, enough O_2 penetrates to start the process of regeneration. The O_2 consumption of these ligatured stems is about the same as for stems which have open ends (Table V). A similar result was obtained by Miller (1937) in comparing regeneration of ends in circulating sea water and standing sea water. More hydranths regenerate when the ends of the stem are bathed with circulating sea water.

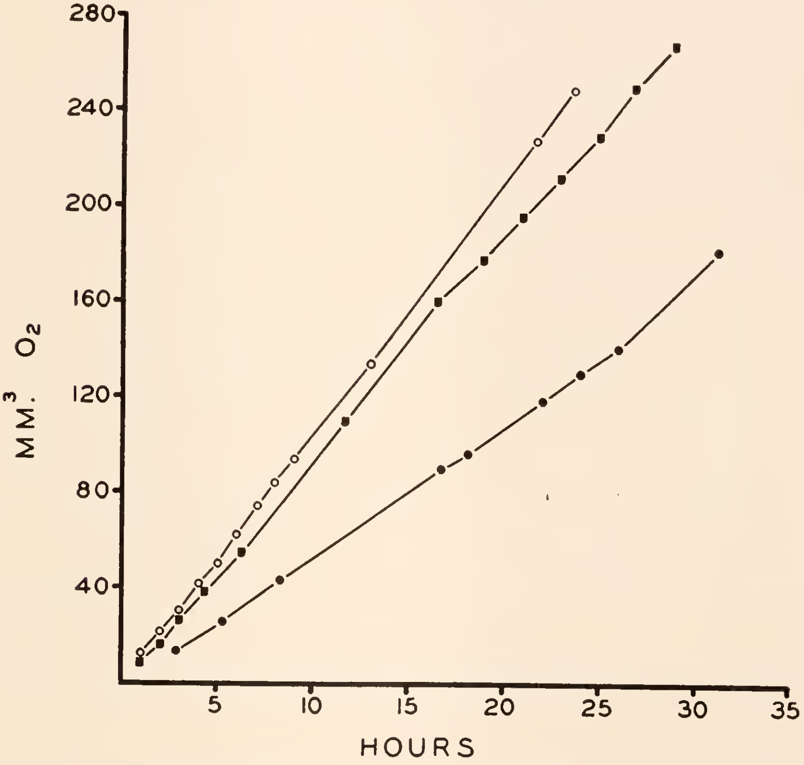

FIG. 2. Oxygen consumption of long stems, plotted against time. Squares = 12 mm. stems; open circles = 10 mm. stems; solid circles = 10 mm. stems. Hydranths fully formed at 30 hours.

Comparison of Rates of O_2 Consumption of Regenerating Stems with O_2 Consumption of Non-regenerating Stems

The rate of oxygen consumption of the regenerant itself must be only slightly greater than that of resting tissue. Attempts to measure the O_2 consumption of the regenerant itself were not very successful. The first attempt was made by ligaturing the stems and comparing the

TABLE IV

Rate of O_2 consumption and rate of regeneration at different O_2 tensions. Gas mixtures passed through vessels for 15 minutes with shaking. Twenty-seven stems (3 mm. in length) having a wet weight of 14 mg. were used for each gas mixture. Temperature 18.45 ± 0.02° C. Oxygen consumption measured for 28.75 hours. Stems then removed and the size of the hydranth measured.

Oxygen mixtures	Rate of regeneration μ^3 hrs. 10^5	Oxygen consumption mm.^{3} O_2/hr./14 mg. wet weight
O_2	177.0	5.4
air	155.0	4.9
1 vol. air to 5 vol. N_2	22.4	3.6
N_2	0	1.6

oxygen consumption of these stems with normal stems. The results are given in Table V. In both experiments the ligatured stems exhibited about the same O_2 consumption as non-ligatured stems. In the first experiment the ligatured stems did not form hydranths yet they consumed about the same amount of oxygen as those which did. The second experiment is complicated by the fact that regeneration occurred even in the ligatured stems.

TABLE V

Comparison of oxygen consumption of ligatured stems with non ligatured stems. Rate = mm.^{3} O_2/hr./10 mg. dry weight.

Description of stems	No.	Length	Rate	Remarks
		mm.		
Ligatured	7	12	31.4	No regeneration
Nonligatured	7	12	30.4	5 distal hyranths 1 proximal hydranth
Ligatured	10	10	38.4	50 per cent regeneration
Nonligatured	10	10	35.0	100 per cent regeneration

The second method of determining the rate of O_2 consumption of the regenerant consisted in a comparison of the oxygen uptake of a whole stem with that of its parts. Thus in Table VI, first experiment, 24 stems 12 mm. long were selected and 12 were cut into 4 pieces and the oxygen consumption of the fourths were measured. The remaining 12 were placed in a manometer vessel at the same time for comparison. In the case of the stems cut into fourths there are eight regenerating ends, while in the whole stem only two. The expectation was a more rapid rate of O_2 consumption with four times as many regenerants. However, Table VI shows that the O_2 uptake is about the same in the whole stem as in the sum of its parts.

Neither of these methods shows a measurable difference between the amount of O_2 consumed by the regenerant and resting stem. However, the curves for O_2 uptake of long stems (Fig. 2) can be interpreted on the basis that in long stems the regenerant is small in comparison with the resting stem and thus any variation in O_2 uptake caused by the regenerant could not be detected. The S-shaped curves for short stems where the amount of regenerant is relatively larger give evidence that the regenerant uses more oxygen than the resting stem (Fig. 1).

Discussion

These experiments support the idea that the tissues that exhibit the higher O_2 uptake regenerate faster than those that use less O_2 (Child and Hyman, 1926; Hyman, 1926). It might be argued that the regional differences in ability to regenerate caused the difference in rate of O_2 consumption. This is unlikely, as in long stems where the regenerating region forms only a small fraction (1/10 or less) of the resting stem the difference in O_2 consumption of distal and proximal halves is present. Since the greater part of these stems is resting tissue, the difference in rate of O_2 uptake must be due to this resting tissue and not to the regenerant. The regenerant would have to consume O_2 at ten or more times the rate of the resting stem in order to produce the observed difference in rate of O_2 consumption of proximal and distal halves. (Table I.)

TABLE VI

Comparison of the rate of oxygen consumption of whole stems with the rate of oxygen consumption of parts of the stem. Rate = mm.^{3} O_2/hr./10 mg. dry weight.

Description of stems	No.	Length	Rate
		mm.	
Distal fourth	12	3	30.6
Second fourth	12	3	22.1
Third fourth	12	3	20.9
Proximal fourth	12	3	19.2
Whole stems	12	12	23.4
Sum of fourths			22.8
Distal half	20	7.5	18.7
Proximal half	20	7.5	11.9
Whole stem	20	15.0	14.8
Sum of halves			15.2

However, since the regenerant consumes only slightly more O_2 than the resting stem, its oxygen consumption cannot account for the regional differences in O_2 uptake measured in distal and proximal parts of stems. Therefore, the gradient in oxygen consumption is an inherent characteristic of the resting tissues at various levels of the stem. The evidence from varying the concentration of O_2 to which the tissues are exposed indicates that as the tissues increase their O_2 consumption they are able to regenerate faster.

It will be of interest in further experiments to see whether the O_2 consumption can be varied without changing the rate of regeneration and also whether the rate of regeneration can be changed without affecting the O_2 consumption. A word of caution is necessary here since in the sea urchin's egg it is quite possible to increase the rate of O_2 uptake of the unfertilized egg until it equals that of the fertilized egg without stimulating the egg to develop. Thus the rate of regeneration and the rate of O_2 consumption may be dependent upon two different processes which thus far are affected by the same treatment and conceivably some treatment might be found where either could be changed independently of the other.

Summary

The rates of oxygen consumption and the rates of regeneration of parts of the stem of *Tubularia* are greater in the distal levels of the stem than in the proximal levels.

The regenerating end of the stem consumes oxygen at a rate which is not much greater than the rate of the resting stem.

In different gas mixtures the rate of oxygen consumption of the stem and the rate of regeneration of the hydranth vary in the same direction.

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BUDS INDUCED BY IMPLANTS OF POSTERIOR NERVE CORD AND NEIGHBORING TISSUES INSERTED AT VARIOUS LEVELS IN CLYMENELLA TORQUATA

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It has been reported that when pieces of nerve cord and neighboring tissues from anterior levels of *Clymenella torquata* (region of segments 1–7) are inserted into various segments of host of the same species the organization of the resulting buds, which frequently develop, is apparently greatly influenced by the host (Sayles, 1940). The orientation of the implant is, however, an important factor. Anterior pieces implanted with their anterior ends in the coelomic cavity and their posterior ends exposed at the surface of the host very rarely produce buds showing definite head or tail characteristics. It was earlier shown (Sayles, 1939) that even though, under some conditions, the nature of the bud might apparently be determined by the host, the new material nevertheless organized so that the exposed part of the implant was in the ventral part of the base of the bud with its original external surface again on the surface.

Implants from the posterior region of donors (segments 13–18) have now been used in 514 operations. In 194 of these the posterior ends of the implants were exposed, while in 311 it was the anterior ends which were at the surface. In 9 early cases no attention was paid to the matter of orientation. All of this experimental work was carried on at the Marine Biological Laboratory, Woods Hole, during the summers of 1935–1939, inclusive. The living material was generously provided by the staff of the Supply Department of the Laboratory.

RESULTS

For all of the following operations the hosts and donors were first narcotized in a chloretone solution made by adding 1 part saturated solution (in sea water) of chloretone to 9 parts sea water. As in previous experiments, the implants were inserted in the dorso-lateral parts of the segments.

In the following discussion the segments of origin of the implants will be given in each case, using the expression "implant 14–15," for example, to mean "implant from segments 14 and 15 of the donor." Except where otherwise stated, the description in each case is of the final condition reached by the bud either at the time of preservation or a brief time before the bud was lost.

Posterior End of Implant Exposed

The results of the 194 cases where the posterior ends of the implants were exposed are summarized in Table I. Although a few of these implants included part of the twelfth segment, the implant always had at least half of the thirteenth segment present. Thus, in no case was the exposed tip of the implant from a level anterior to the middle of the thirteenth segment. One of the interesting points is that, even at the most anterior levels, no head bud resulted from these posterior implants.

Of the 7 tail buds in the first group, 3 were at the *first* segment. One of these buds (Fig. 1), implant 13–14, consisted of about 7 segments showing only on the ventral side beyond the end of the implant. The anal segment was open dorsally. No setae were evident. Another bud, implant 15–16, was short, unsegmented, and with most of the anal segment missing. The small part of the latter present (probably ventral region) terminated in 3 cirri. The third bud (Fig. 2), implant 12–13, included 8 segments, 5 setigerous. The anal segment was open dorsally. On the right side of this segment there was a large, extra, conical mass which showed no evidence of differentiation.

TABLE I

Results of implantation of posterior pieces with posterior end exposed.

Level of insertion segments	1–4	5–7	8	9	10	11	12	13–15	Totals	Percentage of total
Tail + extra part	2	4	1	3		1			11	21.6
Tail buds	5	6		3	7	4	3	3	31	
Indeterminate buds	7	13	1	3	1	2		1	28	14.4
No new material	21	18	2		5	4		1	51	26.3
Died within 10 days	19	29		5	8	2			63	32.5
Implant lost by host	9		1						10	5.2
Totals	63	70	5	14	21	13	3	5	194	100.0

At the *second* segment, one tail bud (Fig. 3), implant 13–14, developed. No setae were present but the possible existence of 6 or 7 segments was indicated by transverse furrows which were confined almost entirely to the ventral side of the bud. Only about one-quarter or one-third of the anal segment was present. This partial segment was at the end of the ventral side of the bud.

At the *third* segment, there was formed one tail bud (Fig. 4), implant 13–14. On the dorsal side there were 2 setigerous segments followed by a short, asetigerous region. Ventrally there was evidence of approximately 7 short segments but no neuropodial uncini. The dorsal half of the anal segment region was missing. Just proximal to this open terminal region there was, at the end of the dorsal half of the bud, a large, undifferentiated, extra part of about the same size as the partial anal cone.

At the *fourth* segment, there were formed 2 large tail buds, each with some of the dorsal part of the anal segment missing. One of these buds, implant 14, included 5 setigerous segments and probably 3 asetigerous ones. The partial anal collar, only about half of which was present, was flattened out into a fan-shaped structure. The other bud (Fig. 5), implant 13, consisted of probably 10 segments, 7 of which were clearly setigerous. In this case only a small part of the anal segment was missing. This segment and particularly the collar and cirri were longest in the mid-ventral region and gradually decreased in extent until they were completely missing mid-dorsally. In each of these cases the bud included the approximate number of segments which one might expect to occur if the determining factor were in the implant.

Of the 10 tail buds found in the region of segments 5–7, one, implant 15, was at the *fifth* segment. This bud was too weakly segmented, even after 30 days, for one to be able to estimate the number of segments included. Only about one-third (ventral part) of the anal segment was present. Sixteen days after the operation a small, club-shaped structure was noted for the first time near the base of the anal segment on the dorsal side. This extra part did not undergo any evident changes during the remaining two weeks which the worm lived.

At the *sixth* segment, 7 tail buds developed. One, implant 12–13, was short and unsegmented. It terminated in what appeared to be a double anal collar region. As this worm died 8 days after the first appearance of new tissue at the implant, no very satisfactory idea of the exact nature of the bud was obtained. Two of these buds, implants 14–15 and 15–16, were unsegmented masses of new material each terminating in a small, partial, anal segment open dorsally (see Fig. 6). Another bud, implant 13–14, showed evidence of possibly several weakly differentiated segments but no setae. The dorsal half of the anal segment was missing. The fifth of these buds, implant 13–14, apparently included about 8 segments, on the basis of constrictions evident only on the ventral side of the bud. Only the ventral part of the anal segment was present. Each of the 2 remaining tail buds formed at this level included an extra part. One of these buds (Fig. 7), implant 13–14, consisted of at least 3 setigerous segments followed by an asetigerous region with the dorsal side of the anal segment region open. On this same side of the bud there was a dorsally directed tongue of material a short distance from the open part. The other bud (Fig. 8), implant 12–13, included 4 setigerous segments followed by an asetigerous region which included a partial anal collar, open dorsally. On the terminal

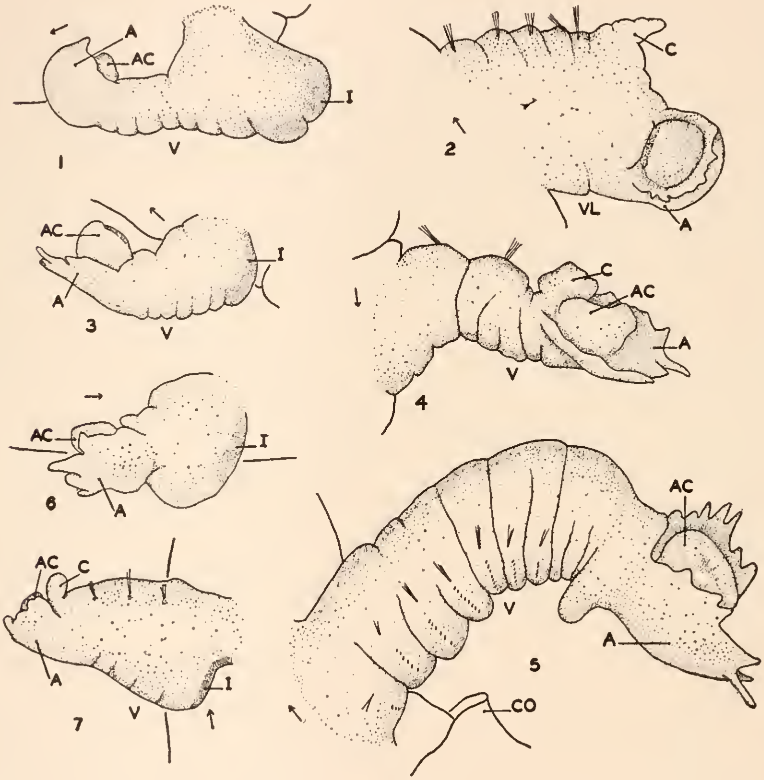

Plate I

Arrow points toward anterior end of host in all figures.

All figures are camera lucida drawings and X22.

Fig. 1. Bud induced at first segment. Age, 18 days. *A*, partial anal segment; *AC*, anal cone; *I*, exposed part of implant; *V*, ventral side of bud.

Fig. 2. Bud induced at first segment. Age, 28 days. *C*, extra cone; *VL*, ventro-lateral region of bud.

Fig. 3. Bud induced at second segment. Age, 12 days.

Fig. 4. Bud induced at third segment. Age, 22 days. *C*, extra part.

Fig. 5. Bud induced at fourth segment. Age, 28 days. *CO*, collar of fifth segment of host.

Fig. 6. Bud induced at sixth segment. Age, 21 days.

Fig. 7. Bud induced at sixth segment. Age, 15 days. *C*, extra cone.

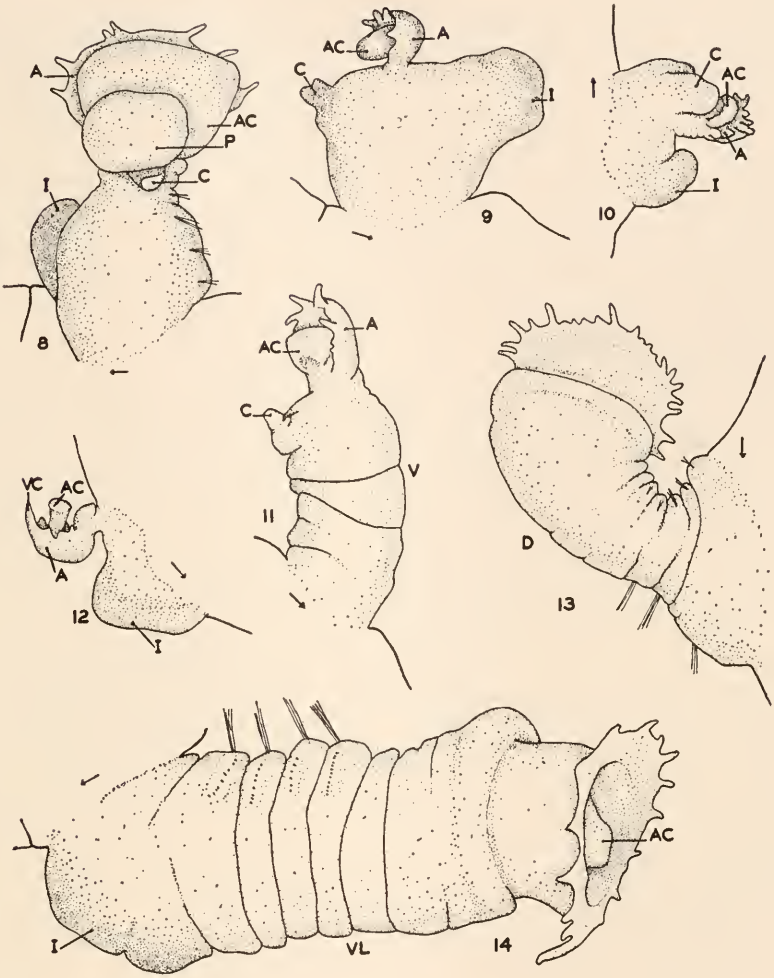

PLATE II

FIG. 8. Bud induced at sixth segment. Age, 23 days. *A*, partial anal segment; *AC*, anal cone; *C*, extra part; *I*, exposed part of implant; *P*, proboscis-like part.

FIG. 9. Bud induced at seventh segment. Age, 30 days.

FIG. 10. Bud induced at ninth segment. Age, 11 days.

FIG. 11. Bud induced at ninth segment. Age, 24 days. *V*, ventral side of bud.

FIG. 12. Bud induced at tenth segment. Age, 19 days. *VC*, long, mid-ventral, anal cirrus.

FIG. 13. Bud induced at tenth segment. Age, 27 days. *D*, dorso-lateral region of bud.

FIG. 14. Bud induced at tenth segment. Age, 33 days. *VL*, ventro-lateral region of bud.

region of the dorsal half of this bud there was a pointed elevation directed dorsally. On the side of this elevation toward the open anal segment there was a rounded part which had something of the appearance and the behavior of a proboscis.

One of the 2 tail buds at the *seventh* segment, implant 12–13, included 6 setigerous segments and probably 3 asetigerous ones, with only the ventral third of the anal segment present. The other bud (Fig. 9), implant 16, was made up of an unsegmented rounded mass possessing one group of notopodial setae and bearing: (1) near the end of the implant, an anal segment region only the ventral part of which was present; (2) on the side of the bud away from the implant, a somewhat irregular part without head or tail characteristics.

At the *eighth* segment, the one tail bud which developed, implant 12–13, was unsegmented, of medium size, and terminated in an anal segment region of which only the ventral third was present. On the dorsal side of the bud, near the anal segment, there was a long, slender process which showed no evidence of differentiation. This entire bud dropped off and was lost without being observed after the seventh day following the appearance of the first distinguishable new material at the implant.

At the *ninth* segment, 6 tail buds developed. Three of these buds, implants 12–13, 12–13 and 13–14, were unsegmented. Each of these 3 terminated in an anal segment the dorsal half of which was missing. Each of the other 3 buds included an anal segment region of which only the ventral portion was present. In addition each of these had a conical elevation at the end of the dorsal half of the main part. Two of these buds, implant 12–13 and 15, were unsegmented (see Fig. 10). The third (Fig. 11), implant 12–13, was weakly segmented.

At the *tenth* segment there developed 7 tail buds none of which had an extra part present. Three buds, implants 12–13, 14 and 15, were unsegmented. In each there was present less than half of the anal segment region, including several anal cirri. These 3 buds were all lost without any records being made concerning which side of the segment was missing. Another bud, implant 14, consisted of 5 or 6 weakly developed segments with the anal one open on one side. The part of this segment present was flattened out into a fan-shaped structure bearing 11 anal cirri. Another bud (Fig. 12), implant 16, was small, unsegmented, slender, and somewhat twisted. The dorsal side of the anal segment region was absent. The remaining 2 buds at this level had complete anal segments. One of these buds (Fig. 13), implant 13, consisted of probably 8 somewhat irregular segments of which the basal 5 were setigerous. The other bud (Fig. 14), implant 14–15, was large and made up of 7 segments, 4 setigerous followed by 3 asetigerous. In this last bud there was the same number of segments as were found in the donor posterior to the source of the implant.

Of the 5 tail buds at the *eleventh* segment, two, implants 13–14 and 14–15, were weakly segmented but terminated in anal segment regions which were complete. In each case the anal collar was longest ventrally and bore a long mid-ventral cirrus. Another weakly segmented bud, implant 15–16, differed from the preceding in lacking the dorsal part of the anal segment. The dorsal half of the bud was truncate. The fourth of these buds (Fig. 15), implant 14–15, consisted ventrally of probably 7 segments. The anal segment and collar were complete. No setae were evident. A long mid-ventral anal cirrus was present, but the others were short. The last of these buds (Fig. 16), implant 13–14, consisted dorsally of at least 3 setigerous segments followed by an asetigerous region. Ventrally there was no evidence of segmentation. The anal segment was complete but with an anal collar which was very short on the dorsal side and without distinct cirri in that region. This anal segment, however, did not occupy the entire end of the bud but was associated mainly with the ventral half of this region. The dorsal half

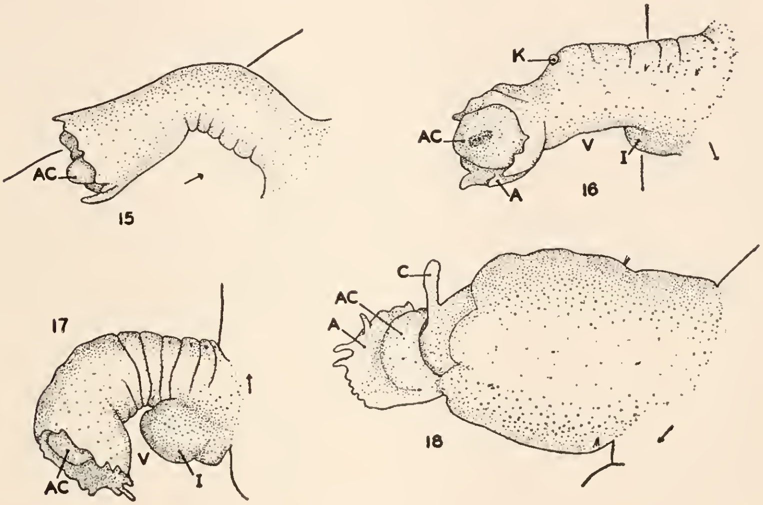

PLATE III

FIG. 15. Bud induced at eleventh segment. Age, 16 days. *AC*, anal cone.

FIG. 16. Bud induced at eleventh segment. Age, 16 days. *A*, partial anal segment; *AC*, anal cone; *I*, exposed part of implant; *K*, club-shaped extra part; *V*, ventral side of bud.

FIG. 17. Bud induced at twelfth segment. Age, 20 days.

FIG. 18. Bud induced at ninth segment. Age, 28 days. *C*, extra part.

of the bud ended in an oblique surface on the extreme dorsal part of which there was a small club-shaped structure. This was the condition 16 days after the implant operation. On the thirteenth day the dorsal third of the anal segment had been missing and the extra part had been present as a cone at the base of the open region. On the tenth day only about one-half of the anal segment had been present and the dorsal half of the bud had terminated in a large rolled edge.

At the *twelfth* segment 3 tail buds were formed. Two of these buds, implants 12–13 and 16–17, were unsegmented. Their anal segment regions were incomplete dorsally. The other bud (Fig. 17), implant 14–15, included, on its dorsal side, 7 segments of which the basal 4 were setigerous. On the ventral side, the implant occupied an unsegmented region corresponding to the basal 3 segments. Ventrally there were apparently 6 or 7 segments beyond the end of the implant. The anal segment was complete but shorter toward the dorsal side than ventrally. There was a long mid-ventral cirrus.

Of the 3 tail buds formed in the region of segments 13–15, 2 were at the *thirteenth* segment. One of these, implant 13–14, was a weakly segmented, long bud with a complete anal segment bearing 13 short cirri. The other bud, implant 14–15, consisted of probably 7 segments and had a complete anal segment and collar.

At the *fifteenth* segment, one tail bud, implant 14–15, developed. It consisted of 7 segments including a complete anal segment bearing a collar of ordinary height ventrally but shorter toward the dorsal region.

The indeterminate buds arising from the implants of posterior pieces did not differ essentially from those previously described as resulting from implants from anterior sources (Sayles, 1940). Three were weakly segmented but with their terminal regions rounded and showing no differentiation. Four were unsegmented, slender buds with rounded tips. Eight were small, undifferentiated cones. Three consisted of small amounts of new material which had developed beneath implant fragments. Ten were irregular masses of new material including one or more parts such as small knobs, one or two notopodia, or scattered neuropodial uncini.

Anterior End of Implant Exposed

The results of 311 operations in which the anterior ends of the implants were exposed are summarized in Table II. In no case was any part of the twelfth segment used in an implant. The most anterior level of an exposed end was, therefore, in the anterior part of a thirteenth segment. No head bud developed. Only one bud which it was deemed safe to interpret as possibly a tail appeared. This one tail bud, implant 13 into segment 4, was of medium size, unsegmented, and with a terminal region which appeared to be a very weakly differentiated anal segment open on one side. This worm died soon after being examined on the fourteenth day after the implant operation.

Of the indeterminate buds, 14 were small cones or slender outgrowths, 2 were small irregular masses of new material, and 8 were new material which had pushed up the implants.

Table II

Results of implantation of posterior pieces with anterior end exposed.

Level of insertion segments	1–4	5–7	8	9	10	11	12	13	Totals	Percentage of total
Tail bud (?)............	1								1	0.3
Indeterminate buds.....	4	1	8	4	5	1	1		24	7.7
No new material........	34	23	29	24	18	1		5	134	43.1
Died within 10 days.....	54	38	26	21	4	2		1	146	46.9
Implant lost by host	1	3	1	1					6	1.9
Totals.................	94	65	64	50	27	4	1	6	311	99.9

Exposed End Unknown

Of the 9 operations for which no record was kept of the orientation of the implant, 4 hosts died within 10 days, 1 lived but produced no apparent new material, and 3 developed small cones or slender outgrowths. In one case, implant 12–13 into segment 9, there developed a large, unsegmented tail bud (Fig. 18) with the anal segment region open dorsally. At the base of this bud there was a single pair of notopodia. Near the open anal segment there was a slender, finger-like outgrowth projecting dorsally.

Discussion

It has been shown (Sayles, 1940) that, following implantation of pieces of anterior nerve cord and neighboring tissues in *Clymenella,* buds of various types may develop. It seems that the determination of the type of bud to be produced in these cases may be controlled through an interaction of host and implant.

The data presented in Tables I and II show that there is a polarity of implants from posterior sources as definite as that shown by those from anterior regions of donors. Although 21.6 per cent of the 194

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implants inserted with their posterior ends exposed produced buds with definite tail characteristics, only 0.3 per cent (1 case) of the 311 implants with anterior ends out produced similar results. The number of cases of indeterminate buds was also much smaller in proportion when the anterior ends of the implants were exposed. On the other hand, the percentage of cases with no evident new material was much greater in the group with the anterior ends of the implants exposed than in that where the posterior ends were out. This polarity is the reverse of that found among implants from anterior sources. The polarities exhibited by the pieces from the two sources are, however, both in accord with ordinary regeneration in *Clymenella* in which definitive buds can be produced only anteriorly in the region of segments 1–7 and only posteriorly at the levels of segments 13–22. Apparently the controlling factors which allow or bring about regeneration in one direction only in these anterior and posterior regions are present in these small implant pieces. This fact in itself does not mean, of course, that these factors are necessarily limited to the nerve cord and its vicinity.

It may also be noted that this polarity of implants gives us an interesting type of control experiment. For example, it might be thought that the occurrence of implant buds is due to a simple mechanical effect of the implants. It was reported (Sayles, 1939) that implanted pieces of intestinal wall or of dorsal body wall either failed to heal into the host or were gradually resorbed without the appearance of any evident new material. Admittedly there might be slight mechanical differences between these different types of pieces. The polarity of these nerve cord implants, however, gives us an excellent control experiment because the same small piece of tissue can induce a bud if oriented one way but not if inserted with the other end out.

It seems clear, therefore, that the implant bears the stimulating factor for bud formation. It also serves as the pathway along which new material migrates to the surface of the host. In the case of implants from posterior sources, as with anterior implants, the bud is organized along the longitudinal axis established by the implant. If the outer tip of the implant heals completely into the body wall of the host in such a way that it is not directed toward the surface of the body, the limited amount of new material which may appear at the surface is restricted in its organization to the possible formation of one or two notopodia or a few scattered neuropodial uncini. The data given in the present paper also add evidence that the orientation of the bud is controlled by the implant. In every case where information is available the implant is found at the base of the ventral side of the induced bud and the cuticle side of any exposed part of the implant is on the surface of the bud.

These experiments, in which implants from posterior sources were used, also show definitely that the determination of the type of bud to be formed is the result of the interaction of implant and host. In the region of segments 1–9 of hosts, implants from anterior sources produce head buds, never tails. Implants of posterior origin, however, form buds the ventral halves of which terminate in partial anal segments (24 cases). In 14 of these tail buds the dorsal portions ended in truncate regions. In each of the other 10 cases there was an undifferentiated, conical or slender part associated with the dorsal region of the end of the bud. One of these dorsal structures (at the sixth segment) was a cone with a proboscis-like part on its ventral side. This was the only case in which any of these buds included a part which might be interpreted as showing a feature characteristic of the head. On the basis of these implants inserted into anterior segments of hosts, it might be thought that there is an inhibitory effect of the host's head which prevents development of a head bud except in the presence of an implant taken from a strongly head-forming region of the donor. That such is not the case is shown by an analysis of the results when implants are inserted at more posterior levels of hosts.

The region of segments 10–12 is a transition zone in the case of each type of implant. Definitive buds arising as a result of implants of anterior origin fall into 4 groups: (1) complete heads, which are common at the tenth segment but rare at the twelfth and thirteenth segments; (2) buds terminating dorsally in partial anal segments and ventrally in structures which may be interpreted as possessing weak head features; (3) buds which terminate dorsally in partial anal segments and ventrally in undifferentiated cones; (4) buds each of which terminates dorsally in a partial anal segment and ventrally in a truncate region at the level of the base of the anal segment. This fourth type is rare at the tenth segment but the predominant type at the eleventh and twelfth segments. On the other hand, buds induced in this mid-body region by implants from posterior sources are of 2 general types: (1) complete tails and (2) buds terminating ventrally in partial anal segments but dorsally in truncate regions. One of the significant points about these buds with partial anal segments is that in buds induced by implants of anterior origin it is the dorsal side of the anal segment which is present while in buds arising from implants of posterior origin it is always the ventral part of the anal segment which develops.

At posterior levels of hosts, the anterior implants produce practically complete tail buds occasionally, but in most cases they form buds terminating dorsally in partial anal segments and ventrally in truncate regions. Not one head bud appeared at these posterior levels. In only one bud was there an extra part in addition to the dorsal part of the anal segment. This extra part was difficult to interpret but suggested, in general appearance and behavior, a proboscis. On the other hand, implants from posterior sources inserted in this posterior region give rise to complete tail buds only.

It seems evident from these results that there is no marked inhibitory influence exerted by the host's head to prevent development of a head bud. If there were such an influence, anterior implants should be able to induce head buds more easily at these posterior levels than in the mid-body or anterior regions. Actually, however, it is in the anterior region that head buds are most readily formed.

Another general point, evident from these experiments, is that the organization of the ventral half of the terminal region of a bud is apparently determined, in most cases at least, by the implant while differentiation of the dorsal half of the bud is under the control of the host. Thus when an implant from an anterior source is inserted into an anterior segment, even as far posterior as the ninth segment, a complete head bud results from the presence of the two combining sets of head-determining factors. Similarly, when an implant from a posterior source is inserted into a posterior segment, a complete tail bud is developed. When, however, anterior implants are inserted at posterior levels, the terminal portion of each definitive bud consists of a truncate region ventrally and a partial anal segment dorsally. The host apparently determines the tail while the implant is relatively ineffective as a determining agent. On the other hand, when posterior implants are inserted at anterior levels the terminal region of each resulting definitive bud is made up of a partial anal segment ventrally and either a truncate region or a conical elevation dorsally. In this group, therefore, the implant apparently determines the tail while the host is comparatively ineffective, although making a slight contribution to the determination of the organization of the bud. In both of these cases it is the tail portion of the bud which is formed and the head-determining factor which is weak. Apparently the determining factor for tail formation present in the posterior region of a *Clymenella* is much stronger than the corresponding factor for head formation present in the anterior region, irrespective of which of these factors is in the implant and which is in the host.

Regarding the number of segments present in the buds, the results are not very definite. In the first place, more data on this point are necessary. Although 42 definitive buds resulted from these implants from posterior sources, segmentation was absent or weak in 24 of them. In 9 others it was impossible to go beyond an estimation of the possible number of segments present. In one case a bud of 7 segments was formed at the fifteenth segment following an implant from segments 14–15 of the donor. The remaining 8 buds indicate that the implant may be more important than the host in determining the number of segments in these tail buds. Implants from 14–15 induced 2 of these buds, both of 7 segments, one developing at the tenth segment, the other at the eleventh segment. Implants from 13–14 or 14 induced 3 buds as follows: one of 7 segments which developed at the first segment of the host; one of 8 segments which was formed at the fourth host segment; one of 8 segments which developed at the sixth segment of the host. Implants from 12–13 or 13 induced the remaining 3 tail buds. These buds were: one of 8 segments at the first segment of the host, one of 10 segments at the fourth segment, and one of 9 segments at the seventh host segment. Although the number of segments in each of these buds was approximately the same as the number posterior to the source of the implant, it should be noted that in every case there were fewer segments than were present in the host posterior to the level of implantation. Since it is common for implants to induce buds which include fewer segments than occur in the corresponding region of the host, this whole matter requires additional study.

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SOME FACTORS WHICH INFLUENCE OXYGEN CONSUMPTION BY BACTERIA IN LAKE WATER[1]

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The conditions which influence the distribution of oxygen in lake water are the subject of an extensive literature (Thienemann, 1928; Grote, 1934), but there is relatively little information on the rôle played by bacteria regardless of the alleged importance of these organisms as agencies which deplete the oxygen content of natural waters. Birge and Juday (1911) state that the respiration of the bacteria of decay is more important than the respiration of aquatic animals in the utilization of oxygen. According to Domogalla, Fred and Peterson (1926) the oxygen content of lake water below the photosynthetic zone is inversely proportional to the abundance of bacteria. Similarly, Kusnetzow and Karsinkin (1931) report that the zones of diminished oxygen tension in Lake Glubokoje are caused primarily by bacterial activity. Seiwell and Seiwell (1938) attribute the oxygen minimum layer in the sea to the increased decomposition of organic matter by bacteria in such zones. Pütter (1924) expresses the view that bacteria consume more oxygen in sea water than all other organisms combined.

Some workers have assumed erroneously that the amount of oxygen consumed by bacteria in water can be evaluated merely by storing samples of raw water in the dark in glass-stoppered bottles and determining the oxygen content after different periods of incubation at the *in situ* temperature. This procedure may serve to estimate the amount of materials in such water which can be oxidized by bacteria but it fails to indicate the rate or the amount of oxygen consumption *in situ* because the storage of water in the laboratory is always accompanied by increased and altered bacterial activity (ZoBell and Anderson, 1936). Therefore, in order to ascertain how much oxygen is consumed by bacteria in lakes, several factors which influence oxygen consumption must be considered.

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[1] This investigation was supported by grants from the Brittingham Trust Fund and the Wisconsin Alumni Research Foundation. Part of the cost of technical assistance was defrayed by the Works Progress Administration.

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Influence of Temperature

Water temperatures ranging from 0° to 35° C. or higher occur in lakes. In order to evaluate the effect of temperature upon the rate at which bacteria consume oxygen, the following experiments were conducted. Water was collected from Lake Mendota in 5-gallon carboys and thoroughly shaken to insure uniformity of its bacterial and chemical composition. It was then siphoned into each of several dozen 145 ml. oxygen bottles. The latter were divided into four lots and transferred to water baths held at 8°, 18°, 25° and 37° C. respectively. Duplicate bottles from each lot were analyzed for the oxygen content of the water by the Winkler method at the beginning of the experiment and after different periods of incubation. In most cases duplicate determinations agreed to within 0.03 mgm./1. and additional bottles were analyzed if the divergence of duplicates exceeded 0.10 mgm./1. The bottles were incubated in the dark to prevent photosynthetic activity. Table I shows the amount of oxygen which was consumed by the bacteria after different periods of incubation.

TABLE I

Amount of oxygen consumed by bacteria in Lake Mendota water incubated at different temperatures. The water initially contained 9000 bacteria per ml. and 7.46 mgm. of oxygen per liter.

Incubation temperature	Milligrams of oxygen consumed per liter after				
	2 days	5 days	10 days	20 days	30 days
8° ± 0.3° C.	0.09	0.26	0.61	1.32	2.84
18° ± 0.3° C.	0.31	1.43	2.41	3.06	3.77
25° ± 0.1° C.	0.56	2.02	2.37	3.95	4.49
37° ± 0.2° C.	0.49	1.84	2.95	4.11	4.76

A multiplicity of correlative factors are involved in the interpretation of the results. In the first place it must be recognized that the rate of reproduction and the death rate of bacteria as well as their respiratory rate are influenced by the temperature of the water. During the first 24 hours the bacterial population of the water incubated at 8° C. had trebled while that incubated at 25° C. had increased nearly a hundredfold. The bacterial population of the water incubated at 25° C. reached its maximum on the third day after which it decreased. The bacterial population of the water incubated at 18° C. reached its maximum on the fourth day and then decreased. The bacterial population of the water incubated at 8° C. was still increasing after ten days at which time it contained more bacteria than the water incubated at the higher temperatures.

The residual organic matter becomes more and more refractory to oxidation as the more oxidizable fractions are oxidized and finally a lack of oxidizable matter limits further oxygen consumption. Although six times as much oxygen was consumed by multiplying cultures in two days at 25° C. as at 8° C., there was virtually no difference after sixty days because at all temperatures most of the readily oxidizable organic matter had been oxidized. By applying the formula of Buchanan and Fulmer (1930):

$$m = \frac{2.303\, S \log b/B}{t(b - B)}$$

where m is the oxygen consumed per cell in time t; S the total amount of oxygen consumed in time t; B the number of bacteria at the beginning of the experiment and b the number after time t; it was found that during the first 24-hour period of incubation at 25° C. the bacteria in Lake Mendota water consumed 21 to 43×10^{-12} mgm. of oxygen per cell per hour. Although the bacterial population continued to increase throughout the second 24-hour period, the oxidizable organic matter content of the water was diminished to such an extent that the bacteria used only 5.4×10^{-12} mgm. of oxygen per cell per hour. After 5 days the bacteria incubated in water at 25° C. were using only 0.5×10^{-12} mgm. of oxygen per cell per hour. During the first 24-hour period of incubation at 8°, 18°, 25° and 37° C., the bacteria consumed an average of 9×10^{-12}, 20×10^{-12}, 32×10^{-12} and 61×10^{-12} mgm. of oxygen per cell per hour respectively. Bacteria from Lake Glubokoje were found by Liagina and Kusnetzow (1937) to consume an average of 18.8×10^{-12} mgm. of oxygen per cell per hour at 10° C. and 30.3×10^{-12} mgm. at 15° C.

The influence of temperature upon the rate of bacterial respiration independent of its effect on bacterial multiplication and other variables mentioned above was investigated by inoculating lake water enriched with 0.01 per cent each of glucose and asparagine with about a hundred million bacteria per ml. The inoculant was prepared by washing the actively multiplying colonies from several bottles of nutrient agar previously inoculated with the mixed microflora from Lake Mendota and incubated at 18° C. Under these conditions the rate of oxygen consumption was found to increase exponentially with the temperature with one exception as shown by Fig. 1. The Q_{10} from 8° to 25° C. was found to be 2.1. The decelerating rate of respiration in the water incubated at 37° C. is attributed to the inactivation of some of the thermo-sensitive bacteria or their enzymes and is not due to a lack of either oxidant or oxygen. According to Edwards and Rettger (1937) the respiratory enzymes of bacteria are especially thermo-sensitive, many being destroyed at relatively low temperatures. Similarly ZoBell and Conn (1940) have shown that prolonged exposure to temperatures exceeding 25° C. is injurious to many water bacteria and that the respiratory enzymes of some are destroyed in 10 minutes at 30° C.

Influence of Oxygen Tension

According to Amberson (1928) the rate of oxygen consumption by unicellular organisms is independent of the oxygen tension over a wide range. Similarly Pütter (1924), Harvey (1928), Pomeroy (1938) and others report that the rate of oxygen uptake by bacteria is not influenced by the oxygen tension. However, Waksman and Carey (1935), Heukelekian (1936), Schlayer (1936) and others find that the rate of oxygen consumption by certain bacteria is proportional to the oxygen tension. These antithetical observations may be due to variations in experimental procedure. For example, Kempner (1937) finds that at relatively low temperatures or with old cells the respiration of *Escherichia coli* is independent of the oxygen tension, but when using

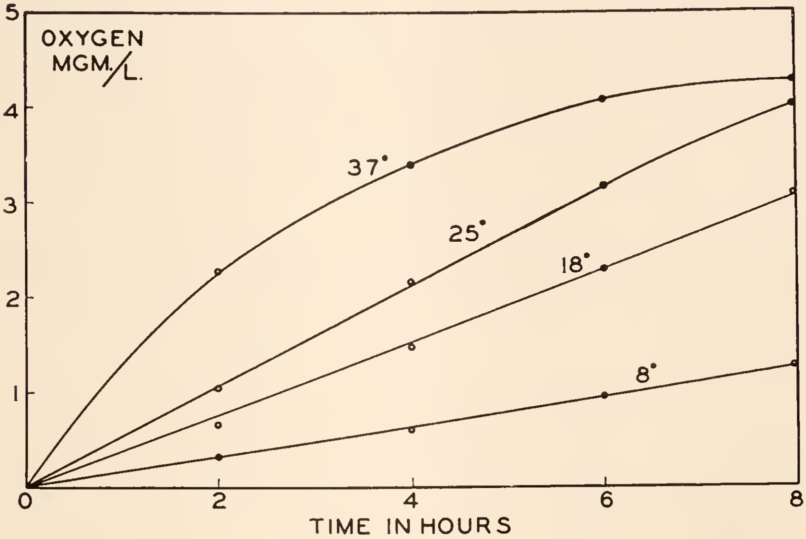

Fig. 1. Oxygen consumed after different periods of time at 8°, 18°, 25° and 37° C. by lake bacteria (resting cells) in lake water enriched with 0.01 per cent each of glucose and asparagine.

heavy suspensions of young cells at their optimum temperature, the oxygen uptake of *E. coli* is a function of the oxygen tension. These observations suggest that all of the oxygen may be consumed in the immediate vicinity of groups or clumps of young cells more rapidly than it can reach them by diffusion. Clifton and Logan (1939) and others have shown that young cells consume oxygen much faster than old ones. According to Waksman and Renn (1936) the effect of oxygen tension is most pronounced when resistant materials are being oxidized.

The following experiments were performed to ascertain what effect the oxygen tension has upon the respiration of bacteria in lake water. The oxygen content of water recently collected from Lake Mendota was adjusted by bubbling either nitrogen or oxygen through it until otherwise identical lots of the water contained from 0.31 to 8.26 mgm./l. This was siphoned into oxygen bottles and the oxygen tension of the water was determined after different periods of incubation at 25° C. The results of the experiment, which are summarized in Table II, show that within the limits of experimental error the oxygen tension of the water does not influence the rate at which oxygen is consumed by respiring bacteria.

TABLE II

Oxygen consumed by multiplying bacteria in Lake Mendota water containing different concentrations of dissolved oxygen after different periods of incubation at 25° C.

Initial dissolved oxygen	Oxygen consumed after				
	1 day	2 days	3 days	5 days	10 days
mgm./l.	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*
0.31	0.21	0.29	—	—	—
0.85	0.23	0.41	0.54	0.79	—
1.78	0.28	0.46	0.61	0.95	1.39
4.02	0.27	0.39	0.50	0.92	1.32
8.26	0.28	0.42	0.56	1.06	1.52

In several other experiments which have been reported in detail by ZoBell and Stadler (1940*a*), it was similarly found that the respiration of "resting" cells in enriched lake water as well as cultures of bacteria is independent of the oxygen tension over a wide range. Although there is a slight tendency for the oxygen tension of lake water to influence the multiplication of bacteria, there is no evidence to indicate that the oxygen uptake of bacteria is influenced by the oxygen tension until the oxygen content is as low as 0.3 mgm./l. Even when the water was supersaturated with oxygen (as much as 26.48 mgm./l.), bacteria consumed oxygen no more rapidly than when the water contained only 0.3 mgm./l. As the oxygen tension is reduced below 0.3 mgm./l. the rate of bacterial respiration decreases rapidly.

Influence of Organic Matter

According to Friedlein (1928) 100 mgm. of utilizable organic matter per liter approximates the minimum concentration in which certain bacteria can grow. This is greatly in excess of the concentration of total organic matter commonly found in lakes; Lake Mendota water containing an average of only 12 mgm./l. (Birge and Juday, 1934); and, as will be shown below, much of this is highly refractory to bacterial decomposition. While many bacteria indigenous to natural waters can maintain themselves indefinitely when sub-cultured in lake water as well as in sea water which contains less than 10 mgm. of total organic matter per liter, this concentration seems to be in the neighborhood of their threshold for multiplication.

TABLE III

Effect of the concentration of glucose upon the rate of respiration of "resting" bacteria in water which initially contained 7.28 mgm. of oxygen per liter.

Concentration of glucose	Oxygen consumed after			
	1 hour	2 hours	4 hours	8 hours
mgm./l.	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*
0	0.06	0.10	0.14	0.23
2	0.12	0.19	0.38	0.53
5	0.16	0.26	0.52	0.91
10	0.17	0.34	0.71	1.18
100	0.29	0.63	1.43	1.69
1000	0.32	0.69	1.38	1.75

The effect of concentration of organic matter upon the rate of oxygen consumption was investigated by adding from 2 to 1000 mgm. of glucose per liter of synthetic lake water inoculated with enough washed bacterial cells (mixed microflora from Lake Mendota) to give around fifty million per ml. After different periods of incubation in oxygen bottles at 25° C. the amount of oxygen consumed was determined. The results are summarized in Table III. Although the "resting" cells were washed twice by centrifuging and re-suspending in synthetic lake water to free them of oxidizable materials, they consumed about 1.0×10^{-12} mgm. of oxygen per cell per hour without the addition of any organic matter. However, the rate of respiration was increased by the addition of glucose until a concentration of 100 to 1000 mgm./l. was reached. Similar results were obtained with glycerol, asparagine and lactic acid. The rate of respiration was not influenced by the addition of 2 mgm./l. of either ammonium sulphate or potassium nitrate thereby proving that the lack of available nitrogen was not a limiting factor. Further experiments are planned to determine the effect of concentration of other organic compounds upon the respiration and other vital activities of bacteria.

TABLE IV

Relative rates of oxidation of 2.0 mgm./l. of organic compounds by cultures of lake bacteria as indicated by the amount of oxygen consumed after different periods of incubation at 25° C. The "oxygen demand" is the amount of oxygen required for the complete (100 per cent) oxidation of 2.0 mgm. of the compound.

Compound	Oxygen demand	Milligrams of oxygen consumed and per cent of each compound oxidized after					
		5 days		10 days		20 days	
	mgm.	*mgm.*	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*
Succinic acid	0.88	0.42	47.8	0.63	71.6	0.83	94.4
Glycine	1.28	0.74	57.8	0.96	75.0	1.16	90.7
Asparagine	1.46	0.59	40.4	0.95	65.1	1.24	85.0
Lactic acid	2.12	1.29	60.9	1.76	83.0	2.10	99.1
Glucose	2.14	1.56	72.9	2.02	94.5	2.07	96.8
Starch	2.36	0.44	18.7	1.19	50.4	2.20	93.3
Cellulose	2.36	0.17	7.2	0.42	17.8	0.96	40.7
Glycerol	2.44	1.63	66.8	1.97	80.8	2.36	97.8
Propionic acid	3.04	0.72	23.7	1.38	45.4	2.24	73.7
Butyric acid	3.64	0.68	18.7	1.49	40.9	2.35	64.6
Lignin	3.70	0	0	0	0	0.23	6.2
Ethanol	4.16	2.06	49.6	3.12	75.0	4.01	96.4

The susceptibility to bacterial attack of different organic compounds which may occur in lacustrine materials was tested by adding 2.0 mgm./l. of the compounds to synthetic lake water (a mineral solution simulating lake water in composition but devoid of oxidizable substrates). The latter was inoculated with mixed microflora from Lake Mendota and distributed in 145 ml. glass-stoppered bottles. In the course of the experiment the bacterial population increased from an initial 26,000 per ml. to several million per ml. after 5 to 20 days incubation at 25° C. Dissolved oxygen was determined at the beginning of the experiment and after different periods of incubation. Some of the data are summarized in Table IV which shows the rapidity with which various compounds are attacked by lake bacteria. Corrections have been made for the 0.07, 0.12 and 0.16 mgm./l. of oxygen which was consumed after 5, 10 and 20 days respectively in the inoculated controls to which no organic matter was added. The "oxygen demand" is the theoretical amount of oxygen required for the complete oxidation of 2.0 mgm. of the compound to carbon dioxide and water. The oxygen demand of the nitrogenous compounds would be somewhat higher if the ammonium resulting from their decomposition were oxidized to nitrite or nitrate.

From Table IV it will be observed that there is considerable difference in the amount of oxygen required for the complete oxidation of similar quantities of different compounds and that there is even a greater difference in the susceptibility of the compounds to bacterial decomposition. Part of the difference in the rate at which oxygen is consumed by bacteria in the presence of different compounds is attributable to differences in the number of bacteria in the mixed culture which are capable of attacking the given compound as well as to differences in the growth-promoting properties of the compounds. Moreover, the fact that the oxidation of some compounds requires four or five times as much oxygen as equal concentrations of other compounds might influence the results. An evaluation of these and other factors which are involved in the full interpretation of the experiment awaits further investigations but the experiment does indicate in a general way how small concentrations of certain organic compounds might influence oxygen consumption by bacteria in lake water. It seems especially noteworthy that 2.0 mgm./l. of some of the compounds are almost quantitively oxidized in 10 to 20 days and the more resistant compounds including cellulose and lignin are slowly oxidized by certain lake bacteria. Working with eleven samples of purified lignin differing either in source or method of preparation ZoBell and Stadler (1940*b*) found that 4.4 to 14.7 per cent of the lignin was oxidized by lake bacteria in 30 days at 28° C. Kinkel (1936) has reported on the decomposition of cellulose, chitin and pectin by aerobic bacteria found in Wisconsin lakes.

Lacustrine Organic Matter

Most of the organic matter which occurs in lake water is quite refractory to bacterial oxidation. This was demonstrated by noting the rate and amount of oxygen consumption in samples collected from various Wisconsin lakes at different times during the year. The water was collected in 5-gallon carboys, filtered through cotton gauze to remove gross particles, thoroughly shaken to insure uniformity in composition and after it was warmed to 25° C., the water was siphoned into 145 ml.

oxygen bottles. Duplicate bottles were analyzed for dissolved oxygen after different periods of incubation in the dark in a water bath at 25° C. Table V shows the oxygen-consuming capacity of Lake Mendota water collected near the surface at Station S 7 from October 11, 1938, to May 6, 1939. In February the samples were collected through twelve to twenty inches of ice.

Assuming the total organic matter content of Lake Mendota water to be 12 mgm./l., the average found by Birge and Juday (1926) over a period of years, and since the complete oxidation of 1.0 mgm. of lacustrine organic matter requires an average of 1.2 mgm. of oxygen, it is estimated that only 21 to 30 per cent of the total organic matter is oxidized in 20 days, and 31 to 37 per cent in 30 days at 25° C. The prolonged incubation of a few samples showed that oxygen continued to be used at a decreasing rate, but after 94 days less than 50 per cent of the organic matter was oxidized. Using the evolution of carbon dioxide as the criterion of decomposition, Allgeier, Peterson and Juday (1934) found that from 18 to 42 per cent of the organic carbon in lake water

Table V

Biochemical oxygen demand (B.O.D.) of Lake Mendota water from Station S 7 after different periods of incubation at 25° C. Upon equilibration with air at 25° C. the oxygen concentration of the water was around 8.5 mgm./l. at the beginning of each experiment.

Description of samples			Oxygen consumed after				Ratio 5:20 day B.O.D.
Date of collection	Oxygen content	Temperature of water	5 days	10 days	20 days	30 days	
	mgm./l.	*° C.*	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*	*per cent*
10/11/38	8.96	16.4	2.06	—	3.84	5.14	53.6
10/25/38	9.08	15.1	2.30	3.82	4.51	5.58	50.9
11/ 2/38	9.37	12.8	2.17	3.73	4.17	—	52.1
11/ 9/38	10.62	8.5	1.54	2.22	3.32	3.96	46.4
11/21/38	11.54	6.6	1.60	2.40	3.41	4.09	46.8
11/30/38	11.93	5.2	0.97	2.07	2.42	3.15	40.2
12/ 8/38	12.45	3.4	0.86	1.65	2.00	2.23	43.0
12/16/38	13.86	1.6	0.96	—	2.14	—	44.4
1/ 4/39	13.08	0	1.36	2.42	3.02	4.21	45.1
1/11/39	14.34	0	1.50	2.31	3.31	4.18	45.4
1/23/39	13.72	0	1.29	—	3.22	3.86	40.2
2/ 6/39	12.90	0	0.91	1.37	2.30	2.60	39.4
2/20/39	12.74	0	0.84	1.21	1.92	—	43.8
3/ 7/39	12.31	0	1.24	1.95	2.99	3.99	41.5
3/23/39	14.18	0.3	3.11	4.66	6.20	7.13	50.2
4/10/39	11.80	7.4	3.56	5.22	7.25	—	49.1
5/ 6/39	10.37	11.6	3.92	5.38	7.46	—	52.5

was oxidized in 31 days at 25° C. According to Waksman and Renn (1936), about 50 per cent of the organic matter in sea water is decomposed by bacteria under similar conditions; and of this decomposed organic matter, about 60 per cent is completely oxidized and about 40 per cent is converted into bacterial cell substance.

Upon the addition of 10 mgm./l. of glucose to the lake water all of the oxygen (about 8.5 mgm./l. at 25° C.) was rapidly depleted from the lake water thereby indicating that available nitrogen is not a factor which limits the oxidation of the organic matter in lake water. Moreover, neither the rate nor the amount of oxygen consumption was influenced by the addition of 2 mgm./l. of ammonium sulphate.

The respirable or oxidizable organic content of Lake Mendota water as indicated by its oxygen-consuming capacity was found to decrease from the time of the fall overturn in October until the ice started to melt late in March (see Table V). The melting of the ice was accompanied by much terrigenous contamination which might account in part for the sudden increase of oxidizable material at this time although other factors, including an increase of phytoplankton, are involved. According to Birge and Juday (1934) the organic matter content of Lake Mendota varies very little throughout the year, centrifuge plankton constituting less than 10 per cent of the total. However, the plankton forms appear to be decomposed much more rapidly and more completely than the resistant residual dissolved organic matter in lake water.

TABLE VI

Oxygen consumed by bacteria (B.O.D.) in samples of water from Northeastern Wisconsin lakes after different periods of incubation at 25° C.

Lake	Date of collection	Oxygen consumed after			Ratio of 5:20 day B.O.D.
		5 days	10 days	20 days	
		mgm./l.	*mgm./l.*	*mgm./l.*	*per cent*
Crystal Lake	1/12/39	0.52	1.34	1.86	28.7
Crystal Lake	2/25/39	0.44	0.95	1.57	28.1
Trout Lake	2/23/39	2.27	3.41	5.33	42.6
Silver Lake	3/29/39	0.83	1.55	2.07	40.2
Little Star Lake	3/29/39	2.12	3.31	5.04	42.1

From 65 to 75 per cent of the organic matter in the net plankton concentrate added to synthetic lake water was found to be oxidized in 20 days at 25° C. Filtering Lake Mendota water collected during the spring months through No. 25 bolting silk which removes the larger plankton forms reduced its oxygen-consuming capacity 4 to 13 per cent.

The Foerst supercentrifuge (Juday, 1926) removed enough particulate organic matter to reduce the oxygen-consuming capacity of lake water 9 to 21 per cent.

The lower the concentration of oxidizable material in lake water the more refractory it is to bacterial attack. For example, from the last of November (11/30/38) to the first of March (3/7/39) when the 20-day B.O.D. of Lake Mendota water was lowest, the ratio of the 5-day B.O.D. to the 20-day B.O.D. was also lowest; or proportionately less oxygen was consumed in 5 days when the total oxidizable content was lowest (see Table V). Similar observations were made on water samples from other Wisconsin lakes as shown in Table VI. Less oxidizable organic matter was found and the ratio of the 5-day to the 20-day B.O.D. was lower in oligotrophic Crystal Lake than in eutrophic Trout, Little Star and Silver Lakes.

TABLE VII

Oxygen consumed per 100 mgm. (dry basis) of Lake Mendota mud dredged from Station 1 in January 1939 after different periods of incubation at 25° C.

Sample No.	Mgm. oxygen consumed after				Ratio of 5:20 day B.O.D.
	5 days	10 days	20 days	30 days	
1380	1.44	2.93	3.67	4.26	39.2
1390	1.25	3.03	4.34	4.63	28.8
1406	1.73	2.78	4.01	4.56	43.1
1409	1.09	1.97	3.58	3.96	30.2
Average	1.38	2.68	3.90	4.35	35.4

The amount of biochemically oxidizable material in Lake Mendota mud was estimated by diluting dredged samples a thousandfold or more with synthetic lake water. The diluted material was equilibrated with air to satisfy any chemical oxygen deficit (Miyadi, 1934), transferred to oxygen bottles with continued shaking to insure uniformity of composition and incubated in the water bath at 25° C. Duplicate bottles were analyzed for oxygen after different periods of incubation. Table VII summarizes the results. The relative slowness with which oxygen is consumed (5:20-day B.O.D. equals 35.4 per cent) shows that the material in the mud is more resistant to bacterial attack than that in Lake Mendota water. Similarly Waksman and Hotchkiss (1938) found that the organic matter in marine bottom deposits is oxidized less readily by bacteria than that in sea water.

According to Black (1929) mud from Station 1 in Lake Mendota contains an average of 13 per cent organic matter (dry basis). Assuming that 1.2 mgm. of oxygen is required for the complete oxidation of 1.0 mgm. of the organic matter, it is calculated that 24 per cent of the organic matter in the lake deposits is oxidized aerobically in 20 days and around 29 per cent is oxidized in 30 days at 25° C. Decomposition continues slowly thereafter at a constantly decreasing rate. The organic matter in the mud is decomposed much more readily in the presence of oxygen than anaerobically because Allgeier et al. (1932) found that only 1 per cent of the total organic carbon was destroyed in 7 months under anaerobic conditions. Steiner and Meloche (1935) have shown that from 30 to 48 per cent of the organic matter in lake deposits consists of ligneous materials which are very resistant.

Little relationship was noted between the oxidizability of the lacustrine materials by permanganate (Wereščagin et al., 1931) and its oxidizability as indicated by oxygen consumption by bacteria. The latter is believed to be much more representative of the capacity of the organic matter to utilize oxygen *in situ*.

Discussion

If the heterotrophic bacteria in Lake Mendota consume oxygen at the rate of 9 to 20×10^{-12} mgm. per cell per hour (values found in the laboratory at 8° and 18° C. respectively), a hundred thousand bacteria per ml. would consume 4 to 9 mgm. of oxygen per liter of water during the six summer months when the temperature of the water ranges from 8° to 18° C. At this rate the activities of heterotrophic bacteria could account for the depletion of the oxygen from stagnant waters particularly in the lake bottom where bacterial populations exceeding a million per ml. have been demonstrated (Henrici and McCoy, 1938). While these estimates based upon laboratory observations are highly speculative, they substantiate the conclusions of field workers that bacteria probably play a very important rôle in the utilization of oxygen in lakes. When more complete data become available it should be possible to evaluate with greater precision the rôle of bacteria. Combining field and laboratory observations Liagina and Kusnetzow (1937) have calculated that " the decrease of the oxygen content of the waters of Lake Glubokoje can be covered with excess by the respiration of the water bacteria."

Although little is known concerning the abundance and activity of chemosynthetic autotrophic bacteria in lakes, those which oxidize ammonium, nitrite, hydrogen sulphide and methane may utilize appreciable quantities of oxygen. The possible significance of such autotrophs is apparent from the fact that the oxidation of 1.0 mgm. of ammonium to nitrate requires 5.15 mgm. of oxygen, the oxidation of 1.0 mgm. of hydrogen sulphide to sulphate requires 4.23 mgm. of oxygen and the oxidation of 1.0 mgm. of methane to carbon dioxide and water requires 4.0 mgm. of oxygen. Hydrographic data (Welch, 1935) indicate the presence of from a trace to a few milligrams per liter of ammonium, hydrogen sulphide and methane in lake water from various places and there is evidence that these substances are oxidized. Kusnetzow (1934) believes that methane-oxidizing bacteria consume more oxygen in certain lakes than all other bacteria and animals combined. According to Ravich-Sherbo (1930) there is an extensive layer of sulphur bacteria in the Black Sea where they oxidize the hydrogen sulphide from the hypolimnion at the expense of the oxygen from the epilimnion. A similar localization of sulphur bacteria in the marginal zone of Lake Ritom has been reported by Düggeli (1924).

Summary

The rate of respiration of lake bacteria increases with temperature, the Q_{10} from 8° to 25° C. being 2.1. Mixed microflora from Lake Mendota were found to consume from 21 to 43×10^{-12} mgm. of oxygen per cell per hour at 25° C. Prolonged exposure at 37° C. is injurious to some of the bacteria.

The rate of respiration is independent of the oxygen concentration of the water within the examined range of 0.31 to 26.48 mgm./1. As the oxygen tension is reduced below 0.3 mgm./1. the rate of bacterial respiration decreases rapidly.

Both the character and the concentration of organic matter influence the rate of oxygen consumption by lake bacteria. The rate of respiration of " resting " cells increases as the concentration of glucose, asparagine and lactic acid is increased up to 100 to 1000 mgm./1.

About one-third of the organic matter in Lake Mendota water is readily oxidizable, the remainder being quite resistant to bacterial attack. The organic content of bottom deposits is less oxidizable than that of water, and the remains of plankton organisms are more readily oxidized by bacteria than the dissolved organic matter occurring in lake water.

Acknowledgment

The author is indebted to Miss Janice Stadler and Dr. F. Cho for technical assistance and to Dr. Elizabeth McCoy, Dr. E. B. Fred, Dr. E. A. Birge and Dr. Chancey Juday for their encouragement and constructive criticisms.

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THE MODIFIABILITY OF THE DIURNAL PIGMENTARY RHYTHM IN ISOPODS

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It has been reported by several observers, among them Kleinholz (1937), that the diurnal variation in the pigment cell dispersion of isopods persists for some days after the animals have been kept in continuous darkness under laboratory conditions. Many other examples of the persistence of diurnal rhythms of one kind or another are given in the reviews of Jores (1935) and of Welsh (1938), the latter also describing some unsuccessful attempts to replace the 24-hour rhythm of animals by a 16-hour one through subjecting them to the influence of "artificial days" (8 hours of light and 8 of darkness). As recently reported (1939), our own experiments on the modification of the diurnal body temperature cycle in man by following a 21- or a 28-hour regime of living were quite successful in some individuals and not in others, indicating a strong individual variation in susceptibility to such modification. Realizing that greater numbers of individuals had to be studied in order to bring out decided group tendencies, it was determined to perform an experiment on animals, and the Bermudan isopod, *Ligia baudiniana,* already studied by Kleinholz, was chosen for the test.

These isopods can be seen in great numbers on the rocky shores near the Bermuda Biological Station and are easily captured. The problem of keeping them alive for more than 2–3 days under laboratory conditions was solved, after some trials, by placing them in individual 150-cc. wide-mouthed bottles, containing a small amount of sea-water and tilted slightly, so that the isopod could remain on either the dry or the wet portion of the bottom of the bottle. A small amount of food in the form of vegetative matter scraped off the wet rocks where the isopods abounded was put into the bottles and replenished as needed. Pieces of cheesecloth were stretched across the mouths of the bottles, permitting air to circulate but preventing the escape of the isopods. Under these conditions some animals remained alive and active for one to two weeks, others for much longer periods.

Only those specimens that were quite dark when captured in the daytime and that showed themselves to be very light during the night following were retained for further observation. They were usually divided at random into two groups, which were placed in separate darkrooms. In one of these rooms darkness prevailed all the time, while in the other light and darkness were alternated automatically by a time clock on an 18-hour cycle of 10 hours of light and 8 hours of darkness. The animals were always observed under uniform conditions of illumination, and a simple system of recording the degree of pigment dispersion was adopted: isopods were classed as definitely dark (score of 2), intermediate (score of 1) or distinctly light (score of 0). The observations were dictated to an assistant, and duplicate observations usually yielded the same scores. The group scores were obtained by adding the individual ones. Thus, a group of 20 isopods might have a pigmentation score as high as 40 and as low as zero. Observations were made 4–6 times per 24 hours, fairly evenly distributed.

The composite data obtained on six groups of isopods, from 16 to 30 to a group, are plotted in curve *A*. It will be seen that after a flattening out of the 24-hour pigmentary activity curve the animals subjected to an 18-hour cycle of light and darkness swung into a distinctly 18-hour pigmentary rhythm. The six separate group curves upon which *A* was based were practically superimposable and were all characterized by three features: (1) an initial period of 24 to 48 hours duration in which the pigmentary rhythm was still definitely diurnal; (2) a transition period, setting in earlier in groups that were started with a period of darkness beginning at noon and later in groups that were first subjected to darkness at dusk (6 P.M.), but in all groups occurring when the repetitive 8-hour shift of darkness commenced at midnight; and (3) a period of 18-hour pigmentary rhythms which may be termed paradoxical in that the greatest pigment dispersion occurred during the artificial darkness and not during the hours of light, as it takes place in nature.

Four groups of isopods, from 12 to 18 per group, were kept in continuous darkness, and these animals preserved their 24-hour cycle of pigmentary activity, as shown in composite curve *B*. They were dark during the daytime hours and pale at night, confirming the observations of Kleinholz. One group was first maintained in continuous darkness, behaving as stated above, and then subjected to the artificial 18-hour cycle. These isopods showed the same three periods of adjustment to the new routine, as did the animals that were under the influence of the 18-hour alternation of light and darkness from the time they were captured.

One group of 20 isopods was maintained under a reversed routine of darkroom illumination (darkness in the daytime and light at night), but it preserved the normal diurnal rhythm of pigmentary activity.

That means, of course, that these isopods were dark during the periods of artificial darkness (daytime) and pale when illuminated (night), resembling both the pigmentary behavior of the animals kept under continuous darkness and the paradoxical pigmentary alternations of the animals that became adjusted to the 18-hour routine.

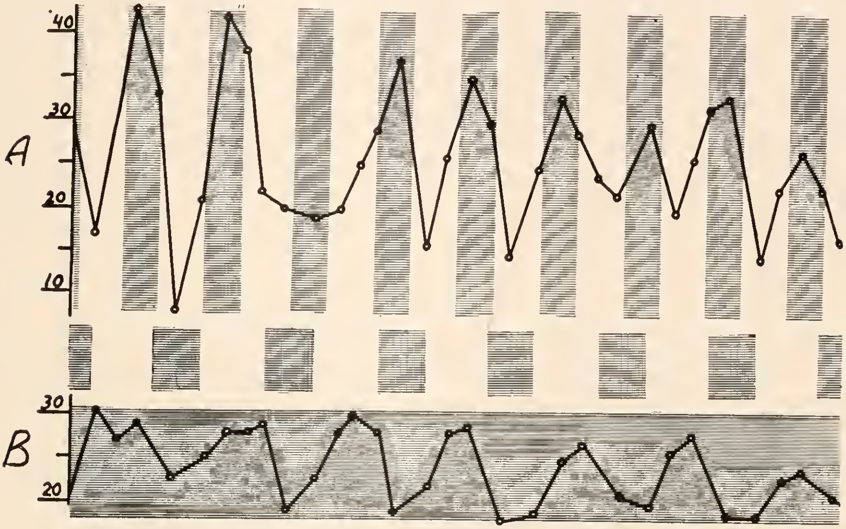

FIG. 1. Periodic variation in pigment dispersion, shown by groups of isopods, kept in the laboratory under artificial conditions of illumination and darkness. Curve *A* is based on the averages of six curves, obtained on groups comprising 30, 30, 20, 20, 16, and 20 animals, respectively. Maximal average pigment dispersion would be plotted as 46, maximum concentration, as zero. The general downward trend of the curve is due to the death of some animals, the total number decreasing from 136 to 104. The alternating areas of gray and white correspond to successive periods of 8 hours of darkness and 10 of light. Curve *B* is based on the averages of four curves, obtained on groups of 13, 12, 18, and 17 animals, respectively. Maximum pigment dispersion is plotted as 30, maximum concentration as zero. As in curve *A*, the downward trend of this curve is due to the death of individual isopods, whose total number decreased from 60 to 50. The gray background of curve *B* indicates the continuous darkness in which these groups were maintained. The alternating areas of gray and white between the two curves correspond to the natural 24-hour succession of night (7 P.M. to 5 A.M.) and day.

Although the occurrence of group pigmentary rhythms is unmistakable, the same cannot be said of individual animals. The groups studied were twice-selected isopods. It will be recalled that only those animals that were quite dark at the time of collection (daytime) were captured, in the first place. From 10 to 25 per cent of the isopods seen on the shore rocks were distinctly pale in the daytime. However, not all the animals that were dark when collected became light-colored during the ensuing night in the laboratory. At least 10–15 per cent remained coal-black at night, and these were also rejected. Thus, the isopods retained for further observation had definitely changed from dark in the daytime to light at night. In the continued manifestation of a pigmentary rhythm the animals in captivity showed marked individual variation. Some isopods closely conformed to the group curves; others were either dark or light most of the time; still others were dark when the group was light and vice versa; finally, there were those whose pigmentary changes followed the group rhythm for one or another portion of the period of observation. As under the natural conditions of alternating day and night, so under the two artificial regimes to which the several groups of isopods were subjected, there were many individual deviations from the general group patterns of rhythmical dispersion and concentration of pigment.

Summary

Although groups of Bermudan isopods, *Ligia baudiniana,* preserve their natural diurnal pigmentary rhythm when kept in total darkness for several days, they rapidly acquire an artificial 18-hour pigmentary rhythm, if exposed to alternating periods of 10 hours of light and 8 of darkness. The new rhythm is paradoxical, pigment cell dispersion occurring during darkness, instead of during the hours of illumination.

I am greatly indebted to Dr. J. F. G. Wheeler, the Director of the Bermuda Biological Station, and to the Staff of the Station, for advice and assistance received during my stay at the Station.

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THE EFFECT OF ALKALINITY UPON MUTUAL INFLUENCES DETERMINING THE DEVELOPMENTAL AXIS IN FUCUS EGGS[1]

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Introduction

When eggs of *Fucus furcatus* develop in the dark in normal sea water in masses they tend to form rhizoids in the resultant direction of neighboring eggs (Whitaker, 1937). This phenomenon, which has been referred to as the "group effect," does not occur when only two eggs are present in a dish unless the medium is acidified. Thus, it has been reported earlier (Whitaker, 1937) that of about 200 eggs reared in pairs (at distances of 4 egg diameters or less) in sea water acidified to pH 6.0,[2] approximately 90 per cent formed rhizoids on the sides toward the neighbor, while in normal sea water at pH 7.8–8.0 only 41 per cent of about 130 eggs did so. Acidification of the medium thus strongly intensifies the "group effect."

It is of some importance in the study of the phenomenon to know whether the strong attraction which exists between two eggs in acid sea water is merely abolished at higher pH, so that each egg is unaffected by the presence of a neighbor, or whether an actual repulsion, or negative "group effect," exists. The fact that less than 50 per cent formed rhizoids toward neighbors at pH 7.8–8.0, in the count repeated above, suggests a negative "group effect," but the numbers are inadequate. Experiments have therefore been undertaken to test this point, extending the pH range as far in the basic direction as possible without much alteration of the composition of the sea water, and with special attention to exactness in pH determination.

Method

Material was obtained and treated in a manner previously described (Whitaker, 1936). Experiments were carried out during March, 1938. The eggs were kept in the dark, or in dim red light, in a constant tem-

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[1] This work has been supported in part by funds granted by the Rockefeller Foundation.

[2] Acidified either with citric acid-secondary sodium phosphate, or with HCl–$NaHCO_3$.

perature room at 15 ± $\frac{1}{4}$° C., from the time of shedding until the results were recorded. The eggs were shed and were fertilized in normal sea water, and were transferred to sea water of artificially altered pH 30–60 minutes after fertilization.

TABLE I

Conditions of the experiments. Eggs were reared two in a dish at distances of 0.3–4 egg diameters, in the dark at 15 ± $\frac{1}{4}$° C. Experiments are arbitrarily numbered in order of pH sequence. The symbol S.W. in column 2 refers to sea water. The pH difference in column 5 shows the change in pH of the medium during the experiment. The results of these experiments are presented graphically in Fig. 1.

Exp. No.	Medium				Total No. Eggs	Rhizoids toward Neighbor per cent
	Composition	pH at start	pH after 24 hrs.	pH change in 24 hrs.		
1	S.W. + KOH	8.85	8.68	−0.17	88	42
2	S.W. + $Na_2CO_3-NaHCO_3$	8.81	8.72	−0.09	74	39
3	S.W. + KOH	8.61	8.48	−0.13	84	29
4	S.W. + $Na_2CO_3-NaHCO_3$	8.58	8.53	−0.05	69	32
5	S.W. + $Na_2CO_3-NaHCO_3$	8.40	8.32	−0.08	119	27
6	S.W. + $Na_2CO_3-NaHCO_3$	8.41	8.36	−0.05	30	23
7	S.W. + KOH	8.42	8.30	−0.12	26	19
8	S.W. + $Na_2CO_3-NaHCO_3$	8.41	8.35	−0.06	18	22
9	S.W. + $Na_2CO_3-NaHCO_3$	8.43	8.34	−0.09	36	22
10	S.W. + $Na_2CO_3-NaHCO_3$	8.22	8.17	−0.05	81	22
11	S.W. + $Na_2CO_3-NaHCO_3$	8.22	8.15	−0.07	68	28
12	S.W. + $Na_2CO_3-NaHCO_3$	8.00	8.12	+0.12	94	38
13	S.W.	8.01	7.93	−0.08	21	38
14	S.W.	8.01	8.09	+0.08	14	36
15	S.W.	7.80	7.70	−0.10	87	37
16	S.W. + citric acid	7.80	7.73	−0.07	52	42
17	S.W. + citric acid	7.61	7.67	+0.06	63	48
18	S.W. + citric acid	7.59	7.65	+0.06	42	52
19	S.W. + citric acid	7.62	7.65	+0.03	38	47
20	S.W. + citric acid	7.58	7.63	+0.05	22	45
21	S.W. + citric acid	7.40	7.43	+0.03	85	56
22	S.W. + citric acid	7.41	7.37	−0.04	32	59
23	S.W. + citric acid	7.41	7.46	+0.05	22	59
24	S.W. + citric acid	7.20	7.27	+0.07	46	76
25	S.W. + citric acid	7.22	7.21	−0.01	60	68
26	S.W. + citric acid	7.21	7.25	+0.04	24	71
27	S.W. + citric acid	6.99	7.10	+0.11	22	86
28	S.W. + citric acid	7.00	7.11	+0.11	62	76
29	S.W. + citric acid	7.02	7.08	+0.06	51	80

The pH of filtered sea water, which was collected at frequent intervals, ranged from 7.8–8.0. Samples were made more basic by adding either KOH, or a mixture of Na_2CO_3 and $NaHCO_3$. Other samples were acidified by adding citric acid. In all cases the osmotic pressure was maintained constant at the time of these additions, and the mixture was vigorously aerated with a large scintered glass nozzle to re-equilibrate with atmospheric CO_2 tension. A glass electrode was used to measure pH, and the aeration was continued until a constant and stable pH was attained. The pH range covered was from 7.0 to 8.8 at intervals of 0.2, as closely as could be approximated. In each experiment pH was measured at the beginning, and also at the end 24 hours later. These pH values, and the composition of the media, are shown in Table I. While the glass electrode was not accurate to the second decimal of pH determination, it approached this accuracy, and therefore the values are recorded to the second decimal as read.

Sea water was not alkalinized much above pH 8.8 because of precipitation of certain salts. There is slight precipitation even at pH 8.8, and at 8.6. The development is altered in the precipitation range so that the rhizoid becomes small and very narrow at the base (Whitaker and Lowrance, 1937) although whether this is due to pH or to loss of salts from the medium is not known.

Eggs were transferred individually into experimental medium in 1 cc. Syracuse dishes by means of a very small pipette. The pipette contained experimental medium which was caused to flow about the egg before it was taken up, and then less than 0.01 cc. solution was carried over with it. Two eggs were placed in each dish at a distance from each other of 0.3–4 egg diameters. The eggs are 65–90 μ in diameter. The dishes were already mounted in moist chambers on a level platform on a vibrationless concrete table.

Results and Conclusions

The results are shown graphically in Fig. 1. Each point in Fig. 1 represents the results of one experiment, and conditions of each experiment are given in Table I.

It is clear from Fig. 1 that a negative "group effect" exists above pH 7.6. This appears to reach a maximum at pH 8.4, when approximately 80 per cent of the eggs form rhizoids on the sides (halves) away from the neighbor. Above pH 8.4 this percentage declines, but since the medium is changing due to precipitation, and the development of the rhizoid is altered, it is not certain that the results above pH 8.4 are entirely comparable. For this reason the curve is dotted in Fig. 1. This evidence of negative "group effect" between 2 eggs in the higher pH range is in agreement with the response of single eggs developing in the ends of capillary tubes in their own diffusion gradients (Whitaker and Lowrance, 1937).

In Table I and Fig. 1 the results are grouped by experiments and pH, without attention to distance between eggs, within the limits 0.3–4 egg diameters. The effect of distance may be seen by dividing the results at a given pH into categories on the basis of distance between eggs.

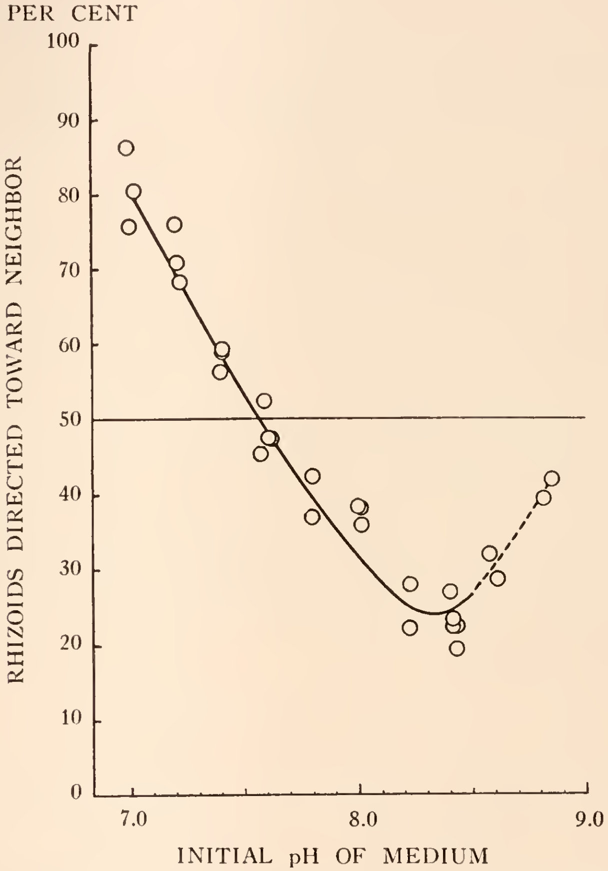

Fig. 1. Results of experiments defined in Table I, showing reversal from positive to negative "group effect" with increasing pH. Each circle represents the results of one experiment, and shows the percentage of eggs which formed rhizoids on the half toward the neighbor. The experiments are grouped at intervals of approximately 0.2 pH unit, with 129–229 eggs at each interval. The curve is dotted through pH 8.6 and 8.8 because of alteration of the medium by precipitation (see text).

At pH 7.0, the percentages of eggs forming rhizoids on the halves toward the neighbor, at different distances, are as follows: 0.3–1 egg diameter, 90 per cent; 1–2 diameters, 75 per cent; 2–3 diameters, 72 per cent; 3–4 diameters, 70 per cent. At pH 8.4 the corresponding figures are: 0.3–1 diameter, 21 per cent; 1–2 diameters, 21 per cent; 2–3 diameters, 31 per cent; 3–4 diameters, 36 per cent. It is seen that within these limits the effect is stronger the closer the eggs, in the pH ranges of both positive and negative "group effect."

SUMMARY

1. When two eggs of *Fucus furcatus* develop in the dark within 0.3–4 egg diameters of each other, the point of rhizoid origin and the developmental axis are influenced by the presence of the neighbor.

2. When the pH of the medium is below approximately 7.6, the rhizoids tend to form on the sides of the eggs toward the neighbor. When it is above 7.6, they tend to form on the sides away from the neighbor, most markedly at pH 8.4.

3. The effect is stronger the closer the eggs, within the limits 0.3–4 egg diameters.

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DEVELOPMENT OF HALF-EGGS OF ARBACIA PUNCTULATA OBTAINED BY CENTRIFUGING AFTER FERTILIZATION, WITH SPECIAL REFERENCE TO PARTHENOGENETIC MEROGONY

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It has been shown previously (1932, 1936) that the half-eggs of *Arbacia punctulata*, which are obtained by centrifuging the *unfertilized* egg, will develop if fertilized or activated artificially. The white halves, containing the ♀ nucleus will develop often quite normally, both fertilized and parthenogenetic, into plutei; the red halves after fertilization (fertilized merogones) containing only the ♂ nucleus will develop, usually in an aberrant fashion, into plutei. The red halves will also develop parthenogenetically (parthenogenetic merogones) as far as the blastula stage, in spite of the fact that they have no nuclei whatever. Additional data with a more complete series of photographs on this subject will be published in a forthcoming number of this journal.

The present paper deals with the development of similar half-eggs of *Arbacia punctulata* which are obtained by centrifuging the *fertilized* egg. It might be expected that these halves would develop similarly to (or better than) those mentioned above, having the same nuclear (or non-nuclear) content. Such has been found, however, not to be the case; they do not develop nearly so well.

Stratification and Breaking of the Fertilized Egg

When *Arbacia* eggs which have been normally fertilized are centrifuged, they stratify into layers similar to the unfertilized egg, only the layers are not nearly so clean-cut (Photograph 1). Except for about five minutes after fertilization, the fertilization membrane prevents the elongation and breaking apart of the egg, so that it is necessary to remove this in order to obtain the half-eggs. This is removed by shaking the eggs 2 minutes after fertilization, just after the membrane has been raised and before it has hardened. The eggs were centrifuged for 6 to 8 minutes at about 10,000 × g., in an isosmotic sugar solution (approximately 2 parts sugar solution to one part sea water + eggs). At one stage of very short duration soon after fertilization, the egg tends to break up into very small pieces when centrifuged, as noted previously (1933); at all other times, it breaks into slightly unequal halves, similar to the unfertilized egg (Photograph 2). At most stages, the white half is slightly larger than the red, but if centrifuged just before cleavage, this half tends to be considerably smaller (Photograph 3). The long streamers which have been described as characteristic of the fertilized egg at a certain stage and which are readily observed with the centrifuge microscope (1933), are preliminary to the final breaking apart and are especially noticeable with relatively low centrifugal forces (5,000 × g.). When the streamers break apart, a sort of tail is often left on the white half; this contracts after 10 to 30 minutes, leaving the white half-egg spherical. The nucleus, which soon after fertilization is the combined ♂ and ♀ nuclei, always goes to the light pole, and is therefore always in the white half, just as is the female nucleus in the unfertilized white half. The ♂ nucleus in the recently fertilized *Arbacia* egg is apparently lighter than the pigment and yolk granules and is not carried by centrifugal force into the heavy half-egg, as it is in the eggs of *Parechinus microtuberculatus, Paracentrotus lividus* and *Sphaerechinus granularis* (1934). It is only in those eggs in which the sperm has entered near the centrifugal end of the egg, as it is thrown into position in the centrifuge, and the egg centrifuged apart while the sperm nucleus is still in this position, that any nucleus is found in the red half-egg which has been completely separated off. This will be described later on. If centrifuged after the amphiaster has been formed, this also, together with the chromosomes, goes to the light pole and is segregated in the white half-egg.

Development of the White Half-egg

The early development of the white half may be fairly normal. The first cleavage divides the egg into two equal cells, through the oil cap (Photograph 4), or through the plane of stratification (Photograph 5) or diagonally; there is a delay of only a few minutes in comparison with the controls. Normal 4 and 8-cell stages follow (Photographs 6, 7), and with further cleavages (Photographs 8–10) a blastula is formed slightly later than the normally developing egg. Owing to the lack of pigment and the small size of the cells, it is difficult to be sure about the micromeres; in a few cases I have thought they were present, but usually they could not be observed. Many of the blastulae, after becoming free-swimming, develop no further though they increase slightly in size—they become "Dauerblastulae," or permanent blastulae (Photograph 11). Some of the blastulae become filled with cells and somewhat differentiated (Photograph 12), some acquire a skeleton often in the form of a primitive triradiate spicule, sometimes more complicated, even without invagination; that is, the formation of the skeleton seems independent of the shape of the larva or other differentiation (Photograph 13). None of the blastulae has developed into a normal pluteus. The nearest approach to a pluteus is shown in Photograph 14; the skeleton approximates the normal, but there are no arms and it is only about a fifth the volume of the normal white pluteus of this age (4 days) from a white half obtained before fertilization and subsequently fertilized. Similar permanent blastulae and imperfect plutei occur together with normal plutei in the white halves fertilized after centrifugation (Photographs in succeeding paper).

Many of the white halves do not develop so normally as this. It has been noted in previous papers (1934, 1940) that the ectoplasmic layer which is formed soon after fertilization and binds the cleavage cells together is thrown off by centrifugal force. In some cases, in the

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Plate I

Development of White Half

The photographs were all taken of living eggs and all brought to approximately the same magnification, 250 ×.

Photograph 1. Fertilized egg centrifuged 4 minutes after fertilization. Photographed immediately; to show stratification.

Photograph 2. Typical red and white halves. Centrifuged 5 minutes after fertilization. Photographed 10 minutes later. Note nucleate white halves, with contracted tail, and non-nucleate reds.

Photograph 3. White half much smaller than red. Centrifuged just before cleavage. Photographed one-half hour later.

Photograph 4. Two-cell stage, cleavage through the oil cap. Centrifuged 11 minutes after fertilization. Photographed 1 hour after fertilization.

Photograph 5. Two-cell stage, cleavage parallel with stratification. Centrifuged 25 minutes after fertilization, streak stage. Photographed 1 hour after fertilization.

Photograph 6. Four-cell stage. Centrifuged 6 minutes after fertilization, monaster stage. Photographed 1¼ hours after fertilization.

Photograph 7. Eight-cell stage. Centrifuged a little before cleavage. Photographed 2½ hours after fertilization.

Photograph 8. Eight to 16-cell stage. Centrifuged 30 minutes after fertilization, streak stage. Photographed 2½ hours after fertilization. Note red halves uncleaved.

Photograph 9. Early blastula. Centrifuged a little before cleavage. Photographed 7 hours after fertilization. Note red half uncleaved.

Photograph 10. White ciliated blastula, 1 day old. Centrifuged 40 minutes after fertilization.

Photograph 11. White ciliate blastula, "Dauerblastula," 2 days old. Centrifuged 15 minutes after fertilization, early streak stage.

Photograph 12. White blastula, 4 days old, somewhat differentiated. Same lot as above.

Photograph 13. Four-day white blastula with triradiate spicule. Same lot.

Photograph 14. Abnormal pluteus, 4 days old, same lot. Most normal pluteus found.

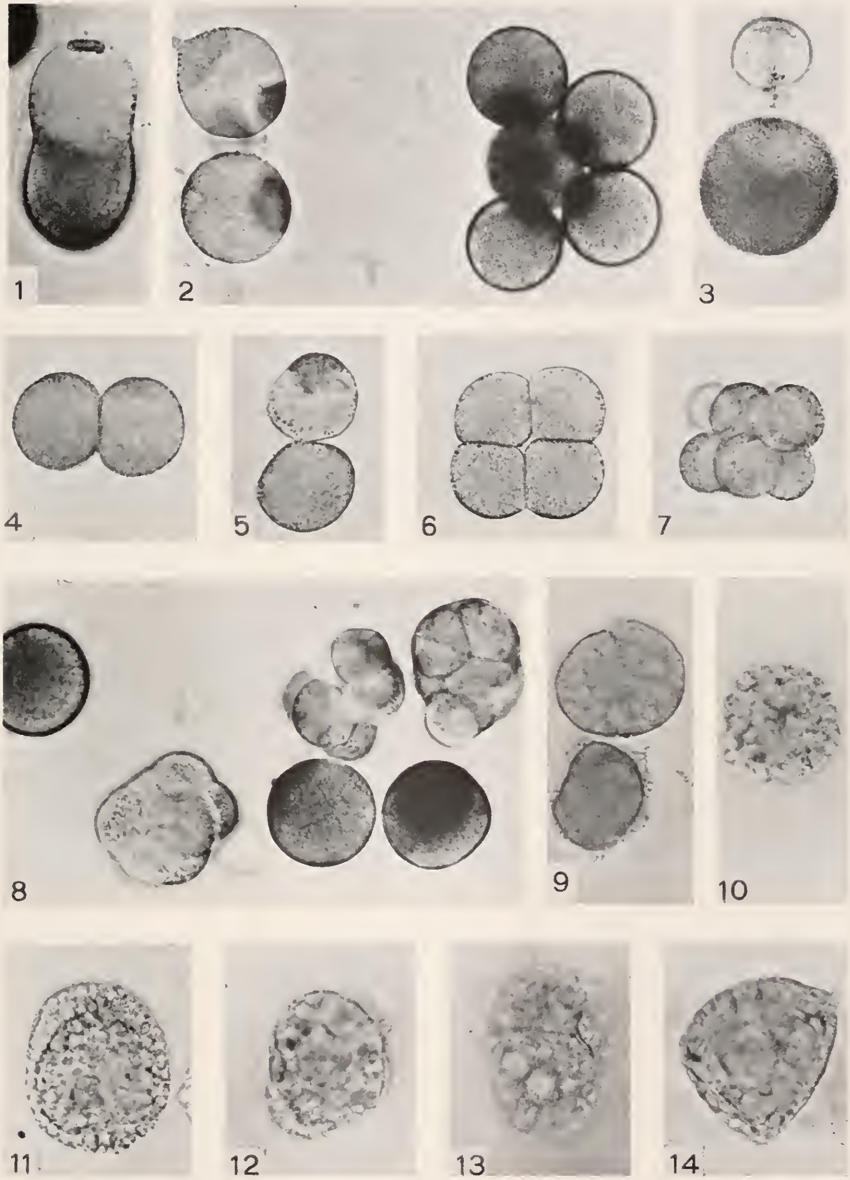
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PLATE I

white halves obtained after fertilization, this layer does not regenerate sufficiently to hold the first two blastomeres together (Photograph 15). Each blastomere develops quite independently, each cleaving in regular sequence to form a white blastula (Photographs 16–21). A pair of white twins is formed which swim at first in pairs quite similar to (but of course smaller than) the twins of the whole egg obtained by centrifuging the fertilized egg at the two-cell stage (1940). These white twins gastrulated, but those isolated had formed no skeleton after 3 days.

Together with these two modes of development of the white halves a less normal development may take place. The white half after having been broken off by centrifugal force, often becomes amoeboid, or it may become amoeboid after several cleavages (Photographs 22–24). It also frequently forms a number of loosely united cells which by further cleavages give rise to a large mass of unorganized cells (Photographs 25, 26). The lack of the ectoplasmic layer is no doubt responsible for this scattering of cells.

Red Half-egg

The red half-egg contains no nucleus but consists of protoplasm which has previously been normally fertilized. These halves often do

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PLATE II

Development of White Half, Continued

PHOTOGRAPH 15. First two white blastomeres remain apart. Centrifuged just before cleavage. Photographed 1½ hours after fertilization. Note red half uncleaved.

PHOTOGRAPH 16. Each white blastomere has cleaved independently. Centrifuged 23 minutes after fertilization. Photographed 1¾ hours after fertilization.

PHOTOGRAPH 17. Each white blastomere is 4-celled. Centrifuged 6 minutes after fertilization. Photographed 2 hours after fertilization.

PHOTOGRAPH 18. Late cleavage of white twins. Centrifuged 40 minutes after fertilization. Photographed 4 hours after fertilization. Note whole blastula below.

PHOTOGRAPH 19. White twin blastulae after becoming free-swimming. Centrifuged 23 minutes after fertilization. Photographed 7 hours after fertilization.

PHOTOGRAPH 20. White twin blastulae, with undeveloped red half. Centrifuged a little before cleavage. Photographed 7½ hours after fertilization.

PHOTOGRAPH 21. A similar pair with whole blastula in comparison. Centrifuged 40 minutes after fertilization. Photographed 7½ hours after fertilization.

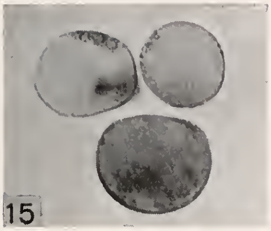
PHOTOGRAPH 22. White half amoeboid. Centrifuged 23 minutes after fertilization. Photographed 1½ hours after fertilization.

PHOTOGRAPH 23. White half very amoeboid. Centrifuged 42 minutes after fertilization. Photographed 1½ hours after fertilization.

PHOTOGRAPH 24. White half amoeboid after several cleavages. Centrifuged just before cleavage. Photographed 3 hours after fertilization. Red half undeveloped.

PHOTOGRAPH 25. White halves form scattered cells. Centrifuged 30 minutes after fertilization. Photographed 2½ hours after fertilization.

PHOTOGRAPH 26. Same group as one shown in Photograph 25, after further cleavages, 1 hour later.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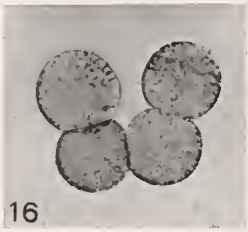
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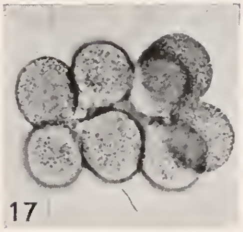
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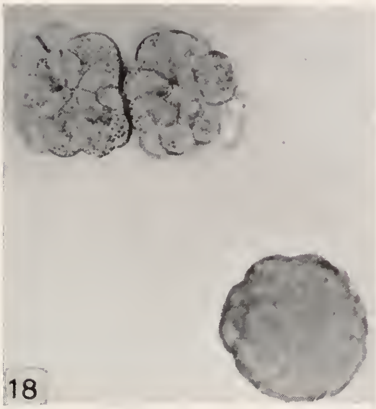
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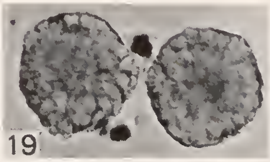
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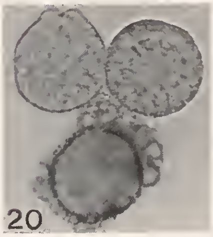
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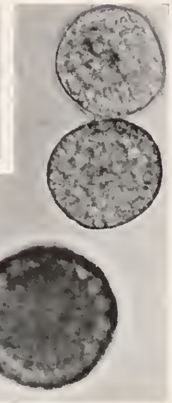
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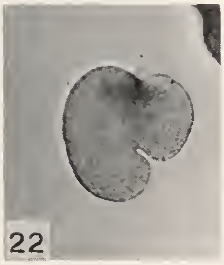
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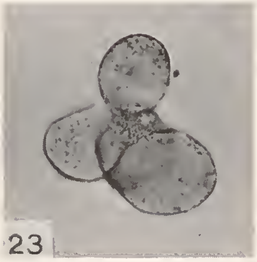
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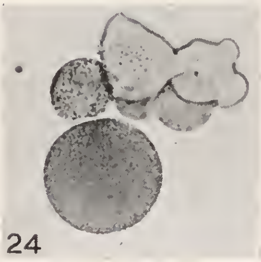
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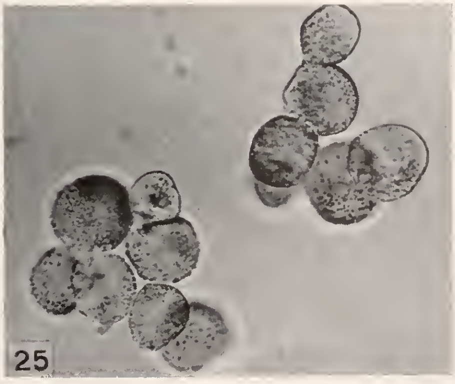
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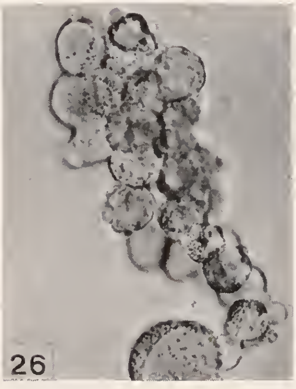
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PLATE II

not change at all even after several hours and do not develop (Photograph 27). In many cases, however, they show signs of activation in that they become amoeboid (Photographs 28, 29). Often a notch appears in the middle of the egg, as though it were about to cleave (Photographs 30, 31). A series of photographs (Photographs 33–36), at intervals of about 10 minutes, indicates that the cleavage does not actually take place, but the notch seems to be an abortive attempt to cleave and soon disappears. The red half sometimes breaks up into a number of small spheres (Photograph 32), but this seems to be by a pinching-off process rather than cleavage as there are no preliminary stages except the amoeboid processes noted above, and the spheres are unstable, that is, they come and go, and they do not become progressively smaller. That the protoplasm is activated and different from unfertilized protoplasm is indicated also by the occasional appearance of a large monaster (Photographs 37, 38), and in one red half a beautiful amphiaster was observed, but this did not lead to cleavage (Photograph 39).

It makes very little difference at what stage after fertilization the eggs are broken apart by centrifugal force. It seemed to me reasonable to suppose that after the nuclear membrane had broken and liberated its contents into the cytoplasm, then this cytoplasm would be different, and the red half would be more likely to cleave than previously. This was, however, not the case. The red halves obtained after the breakdown of the nuclear membrane act exactly like those obtained while the nuclear membrane is still intact; there is no cleavage. This means either that all the achromatic material from the nucleus goes together with the chromosomes and spindle into the light half, or else that it has no effect on the protoplasm, in enabling it to cleave.

PLATE III

Red Halves

PHOTOGRAPH 27. Red halves unchanged after 5 hours. Centrifuged 15 minutes after fertilization.

PHOTOGRAPHS 28, 29. Red halves amoeboid. Centrifuged 30 minutes after fertilization, streak stage. Photographed 4 hours after fertilization.

PHOTOGRAPH 30. Red halves with notch as though ready to cleave. Centrifuged a little before cleavage. Photographed 2½ hours after fertilization.

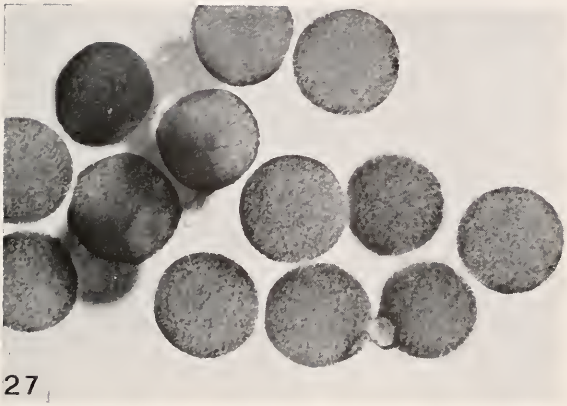
PHOTOGRAPH 31. Red halves notched and amoeboid. Centrifuged 30 minutes after fertilization. Photographed 3 hours after fertilization.

PHOTOGRAPH 32. Red halves pinched into small fragments. Centrifuged 10 minutes after fertilization, monaster stage. Photographed 5 hours later.

PHOTOGRAPHS 33–36. One red half at intervals of 10 minutes. Centrifuged a little before cleavage. Photographed 1½–2 hours after fertilization.

PHOTOGRAPHS 37, 38. Red halves with monaster. Centrifuged 6 minutes after fertilization. Photographed 3 hours later.

PHOTOGRAPH 39. Red half with amphiaster. Centrifuged 21 minutes after fertilization. Photographed 2 hours later.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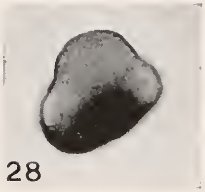
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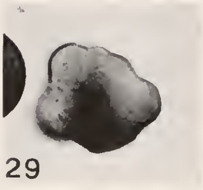
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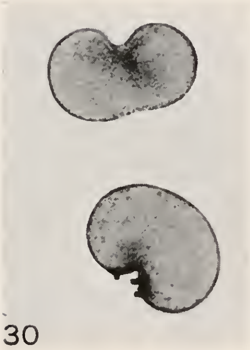
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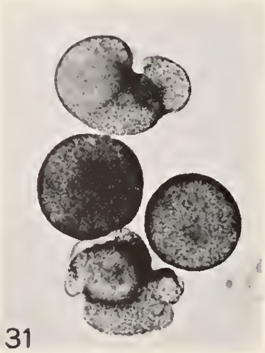
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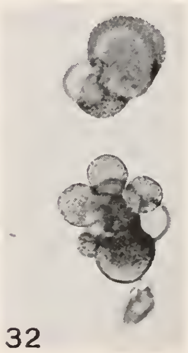
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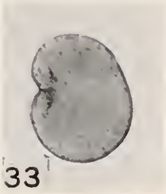
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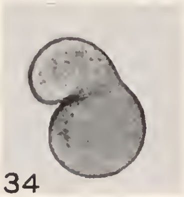
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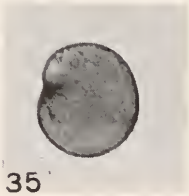
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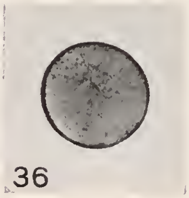
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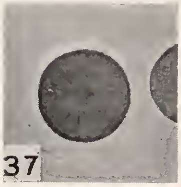
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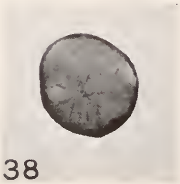
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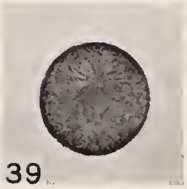
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PLATE III

Re-activation of Halves

The red halves obtained by centrifuging the fertilized egg cannot be re-fertilized nor can they be artificially activated. Even if obtained as quickly as possible after fertilization, re-activation has practically no effect. This is true also of the white halves; there was only a slightly greater tendency toward amoeboid activity and disorganized masses of cells if treated with the hypertonic solution which causes parthenogenesis in the unfertilized halves.

Development of Halves Obtained Immediately After Fertilization

As mentioned above, it is only if one centrifuges the eggs immediately after fertilization, that one can obtain any cleavage in the red halves. This cleavage is due to the presence of the sperm which entered near the centrifugal pole, and the egg has broken apart before the sperm nucleus has had a chance to approach the ♀ nucleus at the centripetal pole. It is well known that the sperm may enter the *Arbacia* egg at any point on the surface, and also that the eggs fall at random in the centrifuge tubes without any orientation. A group of red halves from eggs centrifuged immediately after fertilization is shown in Photograph 40. It is seen in the next photograph (41) that one of these halves contains the ♂ nucleus, and in the two succeeding photographs (42, 43) it is shown that this half-egg cleaves whereas the others do not. If the red half contains the ♂ nucleus, it develops similarly to a

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PLATE IV

Development of Halves Obtained Immediately After Fertilization

PHOTOGRAPH 40. Group of red halves centrifuged off 3 minutes after fertilization. Photographed 1 hour later; monaster in center cell.

PHOTOGRAPH 41. Same group ½ hour later; ♂ nucleus in center cell.

PHOTOGRAPH 42. Same group ½ hour after 41; center cell cleaving. Monaster in egg above to left.

PHOTOGRAPH 43. Same group 20 minutes after 42; center cell completely cleaved, with nucleus in each cell. ♂ nucleus in egg above.

PHOTOGRAPH 44. Development of red half with ♂ nucleus; 3-celled, multinucleate. Same set as above. Photographed 6 hours after fertilization.

PHOTOGRAPH 45. Development of red half, multinucleate. Centrifuged 1 minute after fertilization. Photographed 7 hours later.

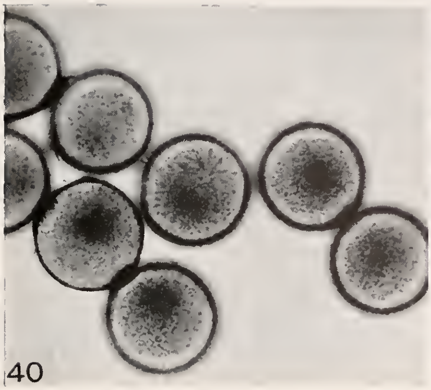
PHOTOGRAPH 46. Group of small pigmented fragments, one with ♂ nucleus. Centrifuged 1 minute after fertilization. Photographed 1 hour later.

PHOTOGRAPH 47. Same lot, another fragment 4 hours later; now 2-celled.

PHOTOGRAPH 48. White half centrifuged 4 minutes after fertilization. Photographed 1½ hours later; normal development.

PHOTOGRAPH 49. White half centrifuged 3 minutes after fertilization. Photographed 3 hours later; normal development.

PHOTOGRAPH 50. White half centrifuged 4 minutes after fertilization. Photographed 3 hours later; disconnected cells.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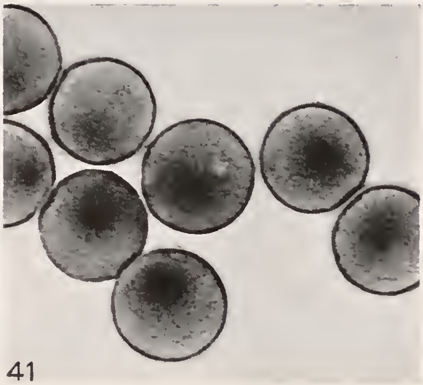
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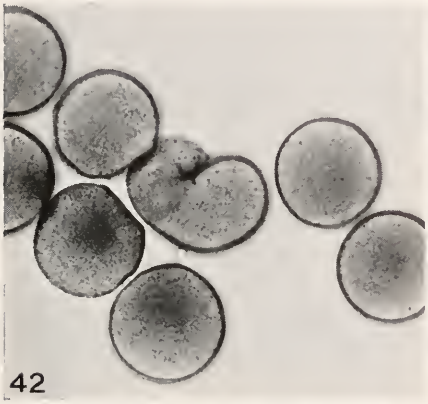
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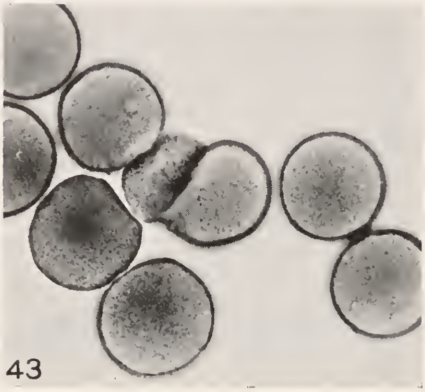
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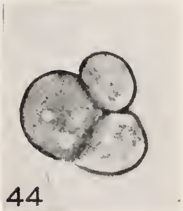
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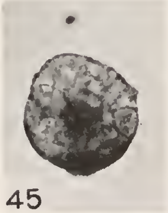
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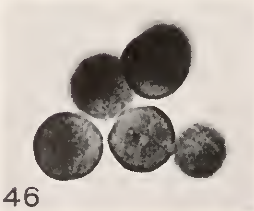
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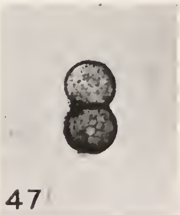
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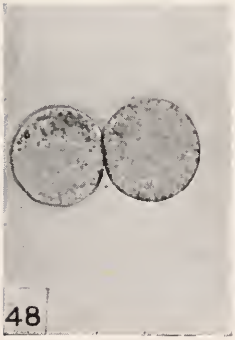
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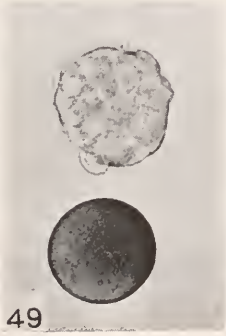
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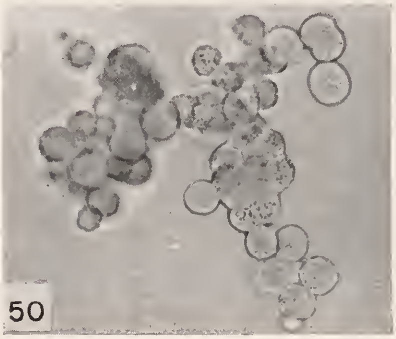
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PLATE IV

red half fertilized after being separated from the white half (i.e. a fertilized merogone), nuclear divisions often taking place without cell divisions (Photographs 44, 45). If centrifuged at a certain period soon after fertilization when the eggs tend to break into small pieces, it occasionally happens that the sperm is entrapped in one of the small heavy fragments (Photograph 46, center cell). This, though quite small, will cleave (Photograph 47); similar fragments without the male nucleus do not cleave.

The white halves obtained by centrifuging the eggs immediately after fertilization, and usually containing both nuclei[1] develop similarly to those broken off at later periods, sometimes cleaving quite regularly (Photographs 48, 49), and sometimes forming irregular masses of disconnected cells (Photograph 50).

Comparison with Parthenogenetic Merogones, and Discussion

The lack of development of the red halves from the fertilized egg is in sharp contrast to the development of the red halves obtained from the unfertilized egg and subsequently activated artificially, the parthenogenetic merogones. These, as I have shown (1936), will cleave in a fairly orderly fashion until they become blastulae. Photographs 51–54 show some successive stages in the development of a group of the parthenogenetic merogones, the unfertilized red halves (51), a group soon after activation (52), and two successive stages in cleavage (53, 54). A few more photographs of fairly normal cleavages are also

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PLATE V

Parthenogenetic Merogones

PHOTOGRAPH 51. Group of red halves obtained by centrifuging the unfertilized egg.

PHOTOGRAPH 52. Similar red halves 3 hours after activation with hypertonic sea water. Note fertilization membrane and ectoplasmic layer, and monaster in lower right egg.

PHOTOGRAPH 53. Similar red halves 4 hours after activation. Early cleavage.

PHOTOGRAPH 54. Same lot of eggs ½ hour later. Further cleavages.

PHOTOGRAPH 55. Four-cell parthenogenetic merogone, 4 hours after activation.

PHOTOGRAPH 56. Eight-cell stage, 4½ hours after activation.

PHOTOGRAPH 57. About 16-cell stage, 7 hours after activation.

PHOTOGRAPH 58. Early blastula of parthenogenetic merogone, 27 hours after activation.

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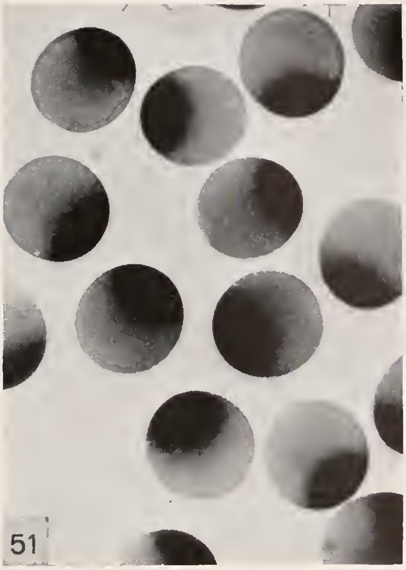
[1] For development of these white halves as watched with the centrifuge microscope see 1933, p. 394; it is difficult to tell whether they have both ♂ and ♀ nuclei or only the ♀ in mass cultures.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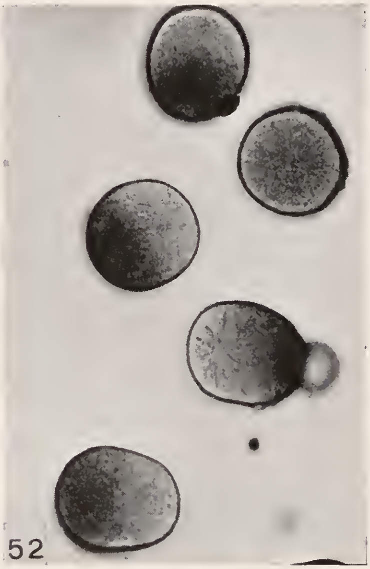
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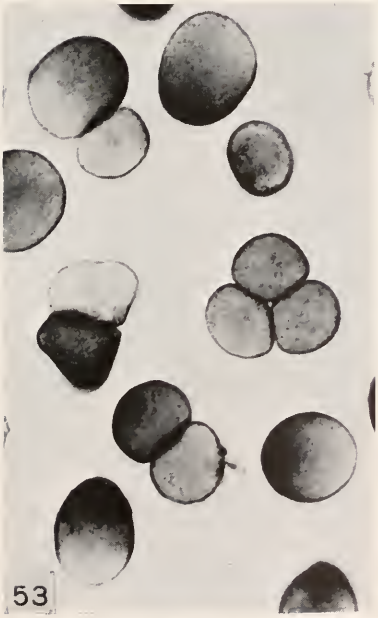
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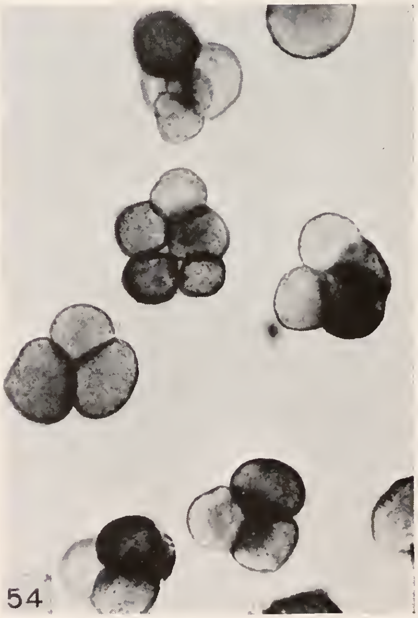
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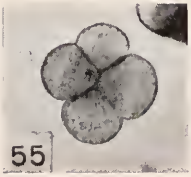
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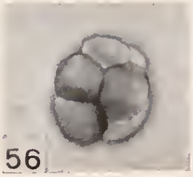
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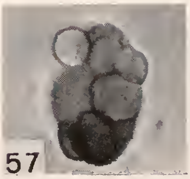
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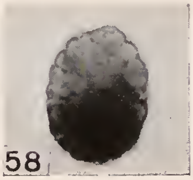
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PLATE V

presented (Photographs 55–58). I wish especially to call attention to the very normal early blastula (Photograph 58).

It seems extraordinary that these red halves which have been activated artificially, without ever having had any contact with sperm or substances from the mature female nucleus, should develop better than the red halves, obtained after fertilization, which consist of protoplasm which has been acted upon by sperm in a normal manner, and which in some cases (when centrifuged after the breakdown of the nuclear membrane) has been mixed with the substances of the nucleus. It might be emphasized here that the fact that the germinal vesicle has liberated substances into the cytoplasm before the occurrence of parthenogenetic merogony has not escaped my notice, and this was particularly discussed in my first paper (1936, p. 119). This in itself cannot have any effect on the development of the parthenogenetic merogone since the other red halves (from eggs fertilized, then centrifuged) do not develop and yet have this material also. The interesting feature of parthenogenetic merogony is that an egg will cleave and by successive cleavages form a blastula, without the presence of the mature ♀ nucleus or any substances from it, and without the ♂ nucleus. The protoplasm of a mature egg is necessarily a product of successive generations and it contains materials of the germinal vesicle from within its boundaries

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Plate VI

Development of Whole Eggs Centrifuged After Fertilization

Photograph 59. Normal 2-cell stage (upper). Egg inside fertilization membrane, in which only white half has cleaved (lower left). Same without fertilization membrane (right). Centrifuged 45 minutes after fertilization. Photographed 2 hours after fertilization.

Photograph 60. Normal micromere stage. Centrifuged just before cleavage. Photographed 2½ hours after fertilization.

Photograph 61. Late cleavage stage, white and red portions distinct. Centrifuged 3 minutes after fertilization. Photographed 4 hours later.

Photograph 62. Blastula soon before hatching. Centrifuged 40 minutes after fertilization. Photographed 6 hours after fertilization.

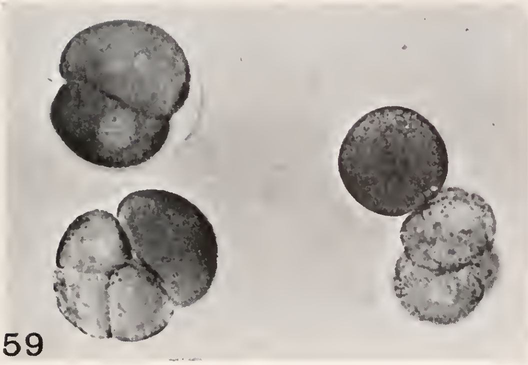
Photograph 63. Whole egg inside fertilization membrane, white portion only developed. Centrifuged just before cleavage. Photographed 6 hours after fertilization.

Photograph 64. Both parts develop with nuclei from original diploid nucleus. Centrifuged just before cleavage. Photographed 8 hours after fertilization.

Photograph 65. Similar egg; two nucleate parts about to separate, forming twins. Centrifuged 30 minutes after fertilization. Photographed 9 hours later.

Photograph 66. Similar pair, 3 days old. Larger one is a white blastula, smaller a red gastrula. Centrifuged 40 minutes after fertilization.

Photographs 67, 68. Two white twins and a red blastula, all with nuclei from original diploid nucleus, forming triplets. Centrifuged soon before cleavage. Photographed 8 hours later.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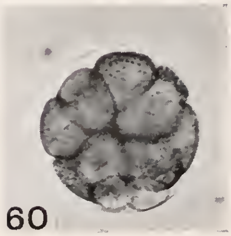
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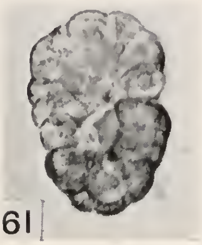
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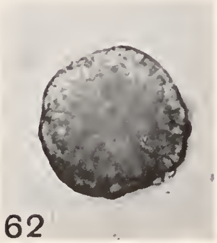
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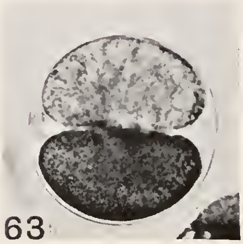
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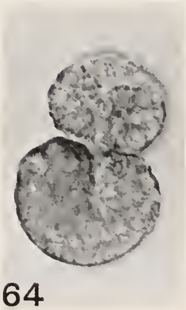
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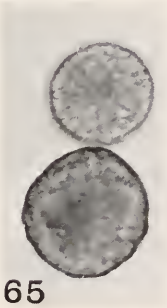
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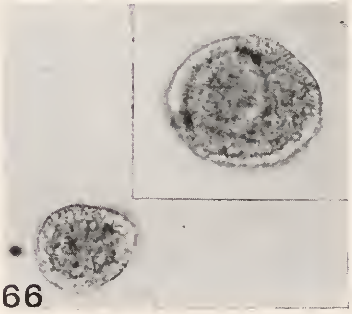
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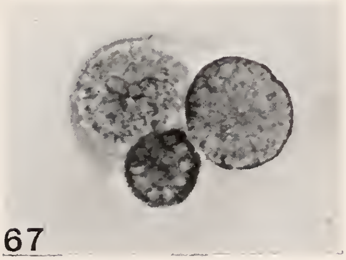
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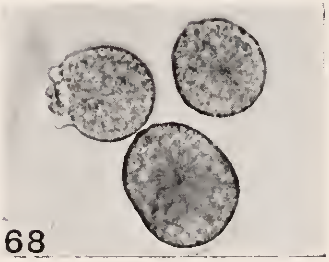
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PLATE VI

as well as nutrient materials from without. Certainly the chromosomes and genes which are generally believed to determine its own fate are *not* present in a parthenogenetic merogone.

Since the non-nucleate half-eggs obtained after fertilization behave so differently from those obtained before fertilization and subsequently activated, it is obvious that a change has taken place in the protoplasm on fertilization which changes its developmental potencies as well as its physical and chemical characteristics such as permeability, viscosity and oxygen consumption. This change must take place very rapidly since the non-nucleate halves will not develop even if obtained as quickly as possible after fertilization nor can they be re-activated. The change in rate of stratification of the fertilized and unfertilized eggs also takes place immediately as well as the change in the rate and method of breaking (1933).

In comparison with the amoeboid activity of the red halves of the *Arbacia* egg, obtained after fertilization, it is of interest to refer to the classical work of E. B. Wilson (1904) on *Dentalium*. He found that the non-nucleate portion, obtained by cutting a fertilized egg, and containing the polar lobe, would go through rhythmic phases simultaneously with the cleavage of the nucleate portion and form a polar lobe just as though it were still a part of the complete egg, and even appeared as though it divided into two.

Development of Whole Eggs Centrifuged after Fertilization

Among the eggs broken apart by centrifugal force, there are always, with the forces used, some eggs which have not broken apart, both with and without fertilization membranes. These may develop quite normally like the uncentrifuged egg, even giving off micromeres (Photographs 59, upper egg; 60). Perfectly normal blastulae and plutei are formed. In some cases the stratification remains during cleavage and the egg may still remain elongate if the fertilization membrane has been removed (Photographs 61, 62), resembling the elongate eggs fertilized after centrifugation. In some cases, even within the fertilization membrane, the two parts, the light and the pigmented, may develop independently, and it frequently happens that the white portion cleaves and the red portion does not (Photographs 59, lower left and 63). If without the membrane, the two portions may be only partially separated and start to develop as a whole, both parts being nucleate (Photograph 64). These parts may become free-swimming blastulae and later separate and give a pair of twins, which are different in color, but with the same nuclear make-up (Photograph 65). Several of these pairs were isolated and in all cases after three days, the white twin was a blastula and the red twin had invaginated (Photograph 66). It would be of interest to know whether these red halves with diploid nuclei would develop better than the fertilized merogones with haploid nuclei which are so difficult to raise. Together with these "twins" occur also "triplets," consisting of a red blastula, and two white blastulae which have apparently developed from the upper portions of the first two blastomeres; these three blastulae are, of course, all nucleate (Photographs 67, 68). My departure from Woods Hole prevented further investigation of these twins and triplets.

Summary

1. Fertilized eggs of *Arbacia* may be broken by centrifugal force into white and red halves similar to the unfertilized egg; the nucleus is in the white half.

2. The white half may develop quite normally through the blastula stage; no normal plutei have been obtained. The first two blastomeres may develop independently forming white twins. Amoeboid forms and loose masses of cells also result from the white half.

3. The red half does not cleave or develop. It may become amoeboid or notched, or form asters, thus indicating activation.

4. It makes little difference at what stage the eggs are centrifuged.

5. The red half cannot be refertilized or activated artificially.

6. Lack of development of the red halves obtained after fertilization is in striking contrast to the development of the red halves obtained before fertilization and subsequently activated artificially (parthenogenetic merogones).

7. Whole eggs may develop normally after centrifuging. They may separate later into 2 or 3 parts, forming nucleate red and white twins or triplets.

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GROWTH AND DIFFERENTIATION OF DAPHNIA MAGNA EGGS IN VITRO

VASIL OBRESHKOVE AND ALAN W. FRASER

(From Bard College, Columbia University and the Marine Biological Laboratory, Woods Hole, Mass.)

It is the purpose of this paper to give an account of the development of *Daphnia magna* eggs *in vitro*. To our knowledge the method presented here of growing parthenogenetic eggs of *Cladocera in vitro* instead of in the brood-chamber of the mother represents the first endeavor in this direction. The study was undertaken because in the course of some ultra-centrifuging experiments two years earlier there arose the need of rearing the eggs of this animal in tissue culture slides. The photomicrographs and the brief description of the developmental stages in the course of the growth of the organism outside the brood-chamber of the mother are intended at this time to serve only as a basis for a more critical attack on a large number of possible problems with *Cladocera* in general.

Methods

The animals employed in this work can be traced to Anderson's stock (1932) and this in turn to animals received from Banta's laboratory. This particular species was utilized previously in our laboratory for a period of about five years for other experimental work. The animals were reared only parthenogenetically and they represented genotypically identical individuals derived originally from a single animal.

A number of different media were tried in an endeavor to grow eggs of *Daphnia magna* successfully *in vitro*. A modified Ringer's solution, utilized by Lévy (1927) for physiological work on isolated organs of *Cladocera*, injured the eggs, and in no case was complete development effected. Likewise an endeavor was made to imitate the cultural condition of the brood-chamber by the introduction of varied amounts of the fluid obtained from the brood-chambers of animals in which there were developing eggs. The growth of eggs in such a medium did not show any advantage over a simpler medium consisting of sterile pond water. This is of considerable interest in view of the nutritive rôle which has been ascribed to the fluid present in the brood-chamber (Dearborn, 1908; Birge, 1918). Contrary to expectation, carefully controlled experiments with eggs reared *in vitro* in a medium consisting of sterile pond water repeatedly showed 100 per cent egg development. Such eggs gave rise to fully developed young in about the same period of time as the eggs developing in the brood-chamber of the mother. These observations establish without any doubt the nutritive sufficiency of materials already stored in each egg at the time of laying, and they also throw some light on the nature and the possible function of the tough membrane which surrounds the developing egg.

A *Daphnia magna* female, the eggs of which were to be removed from the brood-chamber, was allowed to swim for a few minutes in a vessel of pond water which had been rendered sterile by heating (distilled water appears to be injurious to the organism). This procedure freed the animal to some extent from microorganisms which were found to cling to the body and which, when present in the egg medium, interfered with the normal growth of the embryos. The animal was then placed in a depression slide (the type commonly employed for tissue cultures) with a round polished cavity 15 mm. in diameter and 3 mm. deep and the slide was filled with sterile pond water. With the aid of a fine dissecting needle, which was placed over its head, the animal was held in the field of the binocular microscope. Another dissecting needle was applied to the inside of the lower carapace, and the animal was held in place on its side against the surface of the slide. The needle which held the head in place was then transferred to the inside of the upper carapace. The two flaps of the carapace were then spread apart till the upper flap was made to lie against the slide. This procedure exposed the eggs in the brood-chamber. By gently moving the body of the animal back and forth with the dissecting needles, while the flaps of the carapace were still held against the slide, the eggs were made to roll out of the chamber without being subjected to any pressure in the course of the dissection. The remains of the animal were then removed and the water surrounding the eggs was replaced by fresh sterile pond water. The successful removal of eggs from the brood-chamber of the mother without any injury to them determines to a large extent the degree of success in the rearing of the eggs of this animal *in vitro*.

To determine the time required for eggs to complete their development in the brood-chambers of the animals, individual animals were isolated in bottles containing the standard amount of the culture medium (Banta, 1921). On each bottle was recorded the exact time when the eggs were deposited. The animals were reared at 25° C. in a water bath, the temperature of which was controlled to ± 0.01° C. From repeated observations on animals reared under such conditions, it was found that with very few exceptions mothers released their young some time during the forty-sixth hour after the deposition of the eggs.

Many series of developing eggs *in vitro* were carried to completion. The length measurements referred to in this paper and the degree of development of the embryos illustrated in the photomicrographs represent observations on a single clutch consisting of 22 eggs. For other observations made simultaneously with this clutch of eggs, there were carried other parallel series *in vitro* and also eggs developing in the brood-chambers of mothers, serving as controls. In an endeavor to perfect the technic of recording the morphological changes in the course of the development, at least 4 other series of photomicrographs were completed and each one of these was accompanied by other parallel series of eggs growing in depression slides and also developing in the brood-chambers of mothers. *Daphnia magna* eggs when reared *in vitro* were found likewise to complete their embryonic development in about 46 hours at 25° C. It was observed, however, that if the growing eggs were subjected to a lower temperature in the course of routine examination or in the course of replenishing from time to time the fluid in the culture slides, there was a delay of several hours in completing the embryonic development. It was found that whenever a young in the tissue culture slide corresponded in development to one just released from the brood-chamber, it extended its caudal spine which up to this time had been closely adhering to the post abdomen (Fig. 15) and immediately began to exhibit more active swimming movements. The extension of the caudal spine and the more active swimming movements which followed were utilized by us as criteria in judging the end point of embryonic development *in vitro*.

Gross Microscopical Examination of Developing Eggs in Vitro

Inasmuch as the embryos are transparent, the rearing of *Daphnia magna* eggs on tissue culture slides enabled us to make direct observations on some of the sequence of events in the course of development of the embryos without the necessity of staining and sectioning.

The parthenogenetic egg of *Daphnia magna* is enclosed in two membranes. The inner membrane is the vitelline and closely adhering to it on the outside, observable even in eggs a few minutes after their deposition, there is a more prominent membrane. Lebedinsky (1891), in a short account of the development of *Daphnia similis,* refers to this membrane as the chorion. These two membranes exhibit a progressive resistance to mechanical injury with lapse of time from the deposition of the eggs. This was repeatedly demonstrated by subjecting the eggs to ultra-centrifuging at various stages in their development. A comparatively weak centrifugal force consisting of 1800 times gravity was sufficient to rupture the membranes and bring about a disintegration of newly deposited eggs the moment the ultra-centrifuge was started. Eggs three hours after their deposition withstood the same centrifugal force for several minutes without disintegration. Still older eggs were subjected to a centrifugal force of as much as 50,000 times gravity for several minutes without a bursting of the membrane. In our study three-hour-old eggs were usually chosen for growth in tissue culture slides. Such eggs invariably attained 100 per cent development. Earlier eggs may be reared with equal success, but greater precautionary methods must be employed during the dissection of the mother and in the subsequent handling of the eggs.

The first morphological change noticed in the course of development *in vitro* is the appearance of an invagination in eggs nine hours old, reared at 25° C. This invagination gives the first indication of the prospective cephalic region (Fig. 3). It becomes more prominent in the next three hours of development (Fig. 4). At this stage a vertical constriction appears also at the extreme posterior end (lower edge in the figure), which marks the bilateral symmetrical plan of organization which is to follow.

During the first 18 hours of development the eggs remain nearly perfectly spherical and do not show any increase in size. The clutch of eggs studied under very careful control, measured throughout this period 0.275 mm. in diameter. Figure 5 shows an egg 18 hours old. This egg, although it is of the same size as in the previous stages, shows the beginning of the cephalic appendages and also some slight evidence of the beginning of the abdominal appendages.

The prospective left and right halves of the future organism are well marked in embryos 21 hours old (Fig. 6). At this stage the embryos measured 0.337 mm. in length in contrast to 0.275 mm. which characterized the length of all the previous stages up to this point. This represents an initial increase in length of about 60 μ. At this same stage there appears also mid-dorsally at the cephalic region a blastodermic thickening, which constitutes the first external evidence of brain development. The prospective cells which are to form the brain are definitely recognizable earlier in prepared slides. In eggs 24 hours old (Fig. 7) this organ is unmistakable when viewed grossly under the microscope.

In embryos 27 hours old (Fig. 8) there comes into prominence, dorsal to the prospective brain, a mass of granular substance at first grayish in appearance, then, with subsequent development becoming brown and finally black. This granular material, which at first is represented by a single mass, in the 30-hour embryo, is seen to differentiate into two well-established masses now brownish in color, representing the material for the development of the eye (not in focus in the photomicrograph, Fig. 9). This double mass is clearly shown in Fig. 10, representing an embryo 33 hours old. Anterior to the eye masses there is visible also the dense material for the prospective ocellus. The eye in a fully developed individual is represented by a single organ placed in the front of the head, composed of ommatidia. It is interesting to note that this cyclopian eye of *Daphnia magna* has embryologically a double origin. Whether or not the sequence of events involved in the development of this organ has phylogenetic significance remains to be investigated. So gradual and orderly is the fusing of the eye masses into a single structure that we are utilizing this process in our laboratory as a criterion in judging the stage of the development of the embryo *in vitro,* also in predicting the time of the release of the young from the brood-chamber.

The first sign of movement of the body was observed in embryos 30 hours old. This was characterized by slow lateral expansions and contractions of the entire body, resembling massaging motions. There were also observed in the embryo at this stage a few irregular heart

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PLATE I

Photomicrographs of *Daphnia magna* eggs and embryos in the course of their development *in vitro*. Figures 1, 2, 3, 4 represent prints from negatives. Figures 5–12 represent prints made by direct projection of the living material on Defender Velour Black paper utilizing a Bausch and Lomb vertical microscopical slide projector. They are negatives. The magnification is the same throughout. × 117.

FIG. 1. Egg 3 hours after deposition.

FIG. 2. Egg 6 hours old showing the beginning of the formation of a clear zone around the periphery.

FIG. 3. Egg 9 hours old showing the first invagination. The interior light areas in Figs. 1–3 are oil droplets.

FIG. 4. Egg 12 hours old showing the chorion and the first demarcation of the future cephalic portion. A constriction at the extreme posterior end marks the beginning of the bilateral symmetrical plan of development.

FIG. 5. Egg 18 hours old showing further demarcation of the cephalic and abdominal appendages and also a more pronounced revelation of the bilateral symmetrical development.

FIG. 6. Embryo 21 hours old showing further demarcation of the cephalic and abdominal appendages and also a more pronounced revelation of the bilateral symmetrical development.

FIGS. 7, 8, 9. Embryos 24, 27 and 30 hours old respectively, showing further developmental differentiations.

FIG. 10. Embryo 33 hours old, showing the early appearance of the two eye pigment bodies and posterior to them the ocellus, the black bodies surrounded by a clear area, centrally placed in the cephalic region. These bodies are not in focus in the earlier stages.

FIG. 11. Embryo 36 hours old, showing the further differentiation of the two eye masses and the ocellus, the white bodies at the extreme anterior end.

FIG. 12. Side view of an embryo 39 hours old after infolding of the cephalothorax.

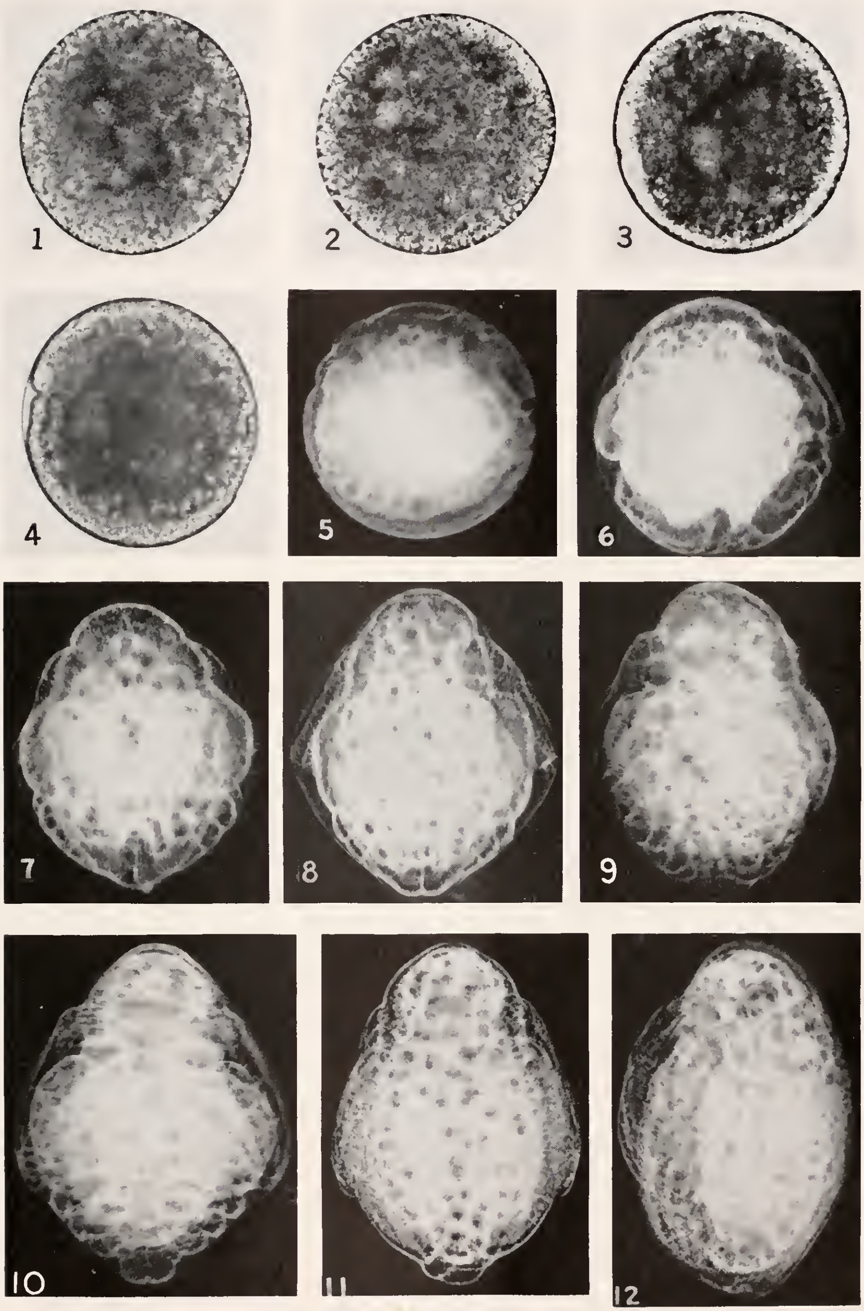
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PLATE I

beats per minute. Five hours later, the heart beats increased to about 20 per minute and in 36-hour embryos the rate became 32 per minute. With the further differentiation of the organism, it became more regular and more rapid. Fully formed young when in their second instar show a heart-beat rate of about 180–200 per minute but this varies greatly with the temperature. The observations recorded here were all made at 19° C. ± 1° C. It is evident, therefore, that the embryonic rate of the heart beat of this organism is less than that in the adult.

The increase in body length in the course of the embryonic development of *Daphnia magna in vitro* is not continuous. There are periods during which no increase in length can be observed, during which time undoubtedly other changes are taking place. As already pointed out, the first increase in body length of about 60 μ was seen in embryos 21 hours old. In the next 3 hours of development there was an additional increase of about 40 μ, and in embryos 27 hours old the body length became 0.41 mm., representing a further growth of about 30 μ over the previous stage. After this there followed a 9-hour period during which the embryos increased in width rather than in length (30, 33 and 36-hour stages). This is more clearly demonstrated in Fig. 10 showing an embryo 33 hours old, just before the infolding of the cephalothorax has begun. After the 36-hour stage and for the next 9 hours there was again observed a gradual increase in body length varying from 20 μ to 60 μ for each 3-hour period of development. The most pronounced increase in body length took place during the hour just previous to the completion of the embryonic development. It appears that with the extension of the caudal spine (Fig. 14) there is also freed a portion of the posterior carapace heretofore held in place. This may account for the apparent sudden increase in the length of the embryo of about 180 μ in one hour (excluding the caudal spine) which is considerable considering the previous increments of growth. This sudden increase in growth may be also due to imbibition of water at this period, a phenomenon so characteristic in fully grown individuals between their successive instars. In some *Daphnia* Miss Rae Whitney of Brown Uni-

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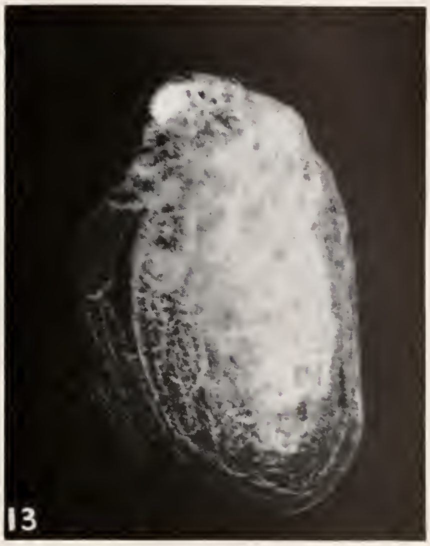
PLATE II

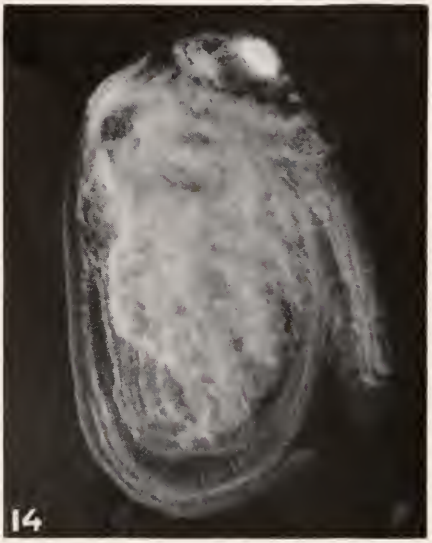
Photomicrographs of *Daphnia magna* embryos in the course of their development *in vitro*, made by direct projection of the embryos on Defender Velour Black paper. They are negatives. The magnification is the same throughout. × 130.

FIG. 13. Embryo 42 hours old showing the eyes laterally placed, and the caudal spine closely adhering to the post-abdomen.

FIG. 14. Embryo 45 hours old—one hour before it emerges as a fully developed young. The caudal spine is only partially extended.

FIG. 15. Embryo 46 hours old representing a fully developed young. The caudal spine is now fully extended.





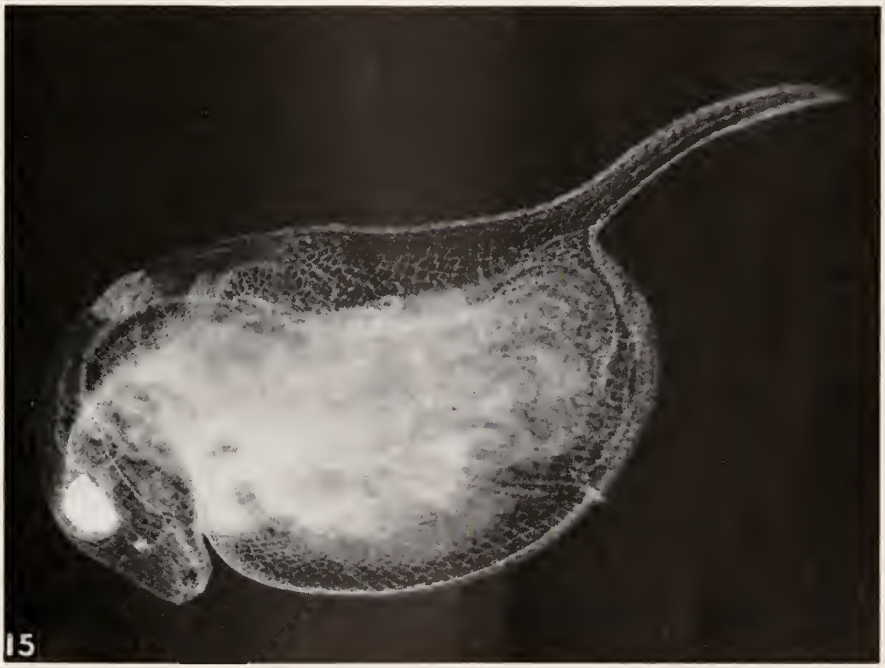


PLATE II

versity finds a pre-natal molt at this stage of the development (unpublished observations).

After the extension of the caudal spine (Fig. 15) the individuals resemble in every respect young just freed from the brood-chambers of mothers. They immediately become more active, utilizing to a fuller extent in their locomotion the antennae and other appendages, thus completing a history of embryonic development from eggs to independent free-swimming organisms in about 46 hours. The individuals at this stage measure about 0.74 mm. in body length. When such individuals are transferred to bottles containing a standard amount of culture medium (Banta, 1921), but at first in a more diluted form, they, too, become mature and in time produce other young.

Summary

1. The parthenogenetic eggs of *Daphnia magna* may be reared in sterile pond water on tissue culture slides, completing their embryonic history from egg to free-swimming independent organisms in about 46 hours.

2. The eggs of this animal are self-sufficient with regard to the nutritive materials already stored in them at the time of their deposition. Such eggs are transformed into fully developed young *in vitro* in about the same period of time as that required for the development of eggs in the brood-chamber of the mother.

3. Fully developed young reared *in vitro,* when transferred to bottles containing the standard amount of culture medium, become in time sexually mature and produce young.

4. A brief description is given of the sequence of events in the course of the development of the embryos reared on tissue culture slides, as observed by gross microscopical examination.

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ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XII. SOME PHYSIOLOGICAL CHANGES IN EGGS THE EMBRYOS OF WHICH HAVE BEEN DESTROYED BY X-IRRADIATION [1]

JOSEPH HALL BODINE, LOREN D. CARLSON AND
O. MALCOLM RAY [2]

(From the Zoölogical Laboratory, State University of Iowa)

Investigations have been conducted on the effect of x-irradiation on the morphology, respiration and enzyme (protyrosinase) formation in the egg of the grasshopper, *Melanoplus differentialis* (Evans, 1934; 1935; 1936; 1937; Boell, Ray and Bodine, 1937; Ray, 1938). By the fifth day of development at 25° C. differentiation has progressed to such a degree that the embryo may be dissected from the egg (Slifer and King, 1934). An x-irradiation of 1000 r given the egg on the sixth day after laying will cause the destruction of the embryo proper leaving the yolk and serosa cells intact (Evans, 1936; Ray, 1938). This permits a study to be made on some of the physiological activities of these eggs from which the embryo has been thus removed.

The following three criteria of physiological activity in these eggs were used: (1) Respiration: The respiration of the whole egg as well as of the contained embryo has been reported (Bodine and Boell, 1936). For the first 15 to 20 days after laying, the rate of O_2 uptake increases; then it declines to a minimum by the twentieth to twenty-fifth day and remains constant during the blocked or diapause state. This period of inactivity (diapause) may be terminated by exposure to 5° C. and when the eggs are again placed at 25° C. there is a rapid increase in the rate of respiration until the grasshopper hatches on the eighteenth to twentieth day. The respiration of the embryo is, at first, lower than that of the intact egg, but later it accounts for a considerable part of late prediapause and postdiapause respiration (Bodine and Boell, 1936). (2) Formation of the enzyme protyrosinase: This enzyme in the normal egg begins to form on the tenth day and by the twentieth day has reached a maximum which is maintained during further development (Bodine, Allen and Boell, 1937). The remarkable resistance of this enzyme to x-irradiation during its formation has been reported (Ray,

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[1] Aided by a grant from the Rockefeller Foundation for work on physiology of the normal cell.

[2] Now at the State Agricultural College of North Dakota.

1938). (3) The ontogenetical change in potency of the naturally occurring activator of protyrosinase: This has recently been investigated by Bodine and others (1939).

MATERIAL AND METHODS

Experiments have been carried out during three successive years, 1937 to 1940, and different lots of eggs used each year. In each lot the eggs were collected on the day of laying, carefully selected and divided into two groups. One of these was irradiated on the sixth day of development; the other kept as a control. The roentgen apparatus used was a double cross-arm mechanically rectified unit energizing a Coolidge broad focus universal air-cooled tube at 130 kv.; and 5 ma. The eggs were spread on several layers of damp towelling and irradiated at a distance of 53 cm. for 20 minutes. Previous calibration with an ionization chamber showed a delivery of 52 r per minute at this distance giving the eggs a total dosage of 1040 r.[3]

The eggs were kept on moist sand at 25° C. during development and placed at 5° C. for 3 months to terminate the diapause. Control eggs were examined at various stages and sorted according to morphological development as reported by Slifer (1932). Care was taken to give the irradiated eggs the same treatment as control eggs. Daily examinations were also made of the experimental material to determine possible injury.

Oxygen consumption of control and irradiated eggs was determined by placing the eggs in lots of 25 or 50 on moist filter paper in standard micro-Warburg manometers.

Tyrosinase activity was measured in egg brei. This was prepared by grinding 100 eggs in a glass mortar and diluting to a volume of 5 cc. with a NaCl solution buffered to pH 6.8 (50 cc. 0.45 per cent NaCl — 25 cc. M/15 Na_2HPO_4 — 25 cc. M/15 KH_2PO_4). The amount of enzyme in this brei was determined in standard Warburg manometers by activating the enzyme with 0.1 cc. 5 per cent sodium oleate and using 0.3 cc. 0.4 per cent tyramine hydrochloride as a substrate. The activity of the enzyme was determined from the rate of oxygen uptake and expressed as $1/T \times 10^3$ where T is the time of half oxidation of the substrate (194 μ l. O_2).

The amounts of natural activator occurring during ontogenesis were determined by the "brei method" described by Bodine et al. (1939). In those cases where the protyrosinase content was low more was added in the form of a standard protyrosinase solution termed B_1 (Bodine et al., 1939). The amount of activation is represented in terms of rate as shown for tyrosinase activity.

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[3] The authors are indebted to Dr. T. C. Evans for x-raying eggs and for making cell counts.

Results

Morphological changes in the eggs were followed daily by removing 10 to 20 eggs at random and inspecting them after dissection under a widefield binocular microscope. In no case was a formed embryo found in the irradiated eggs. By use of smears of the egg contents counts of yolk and serosa cells of both control and irradiated eggs were made. No appreciable differences in appearance or cell counts of experimental and control eggs were detected. Traces of embryonic cells in irradiated eggs occur only for a short period following irradiation. From both gross and microscopic examination one may conclude that the visible effects of irradiation are confined to the embryo proper and not to the

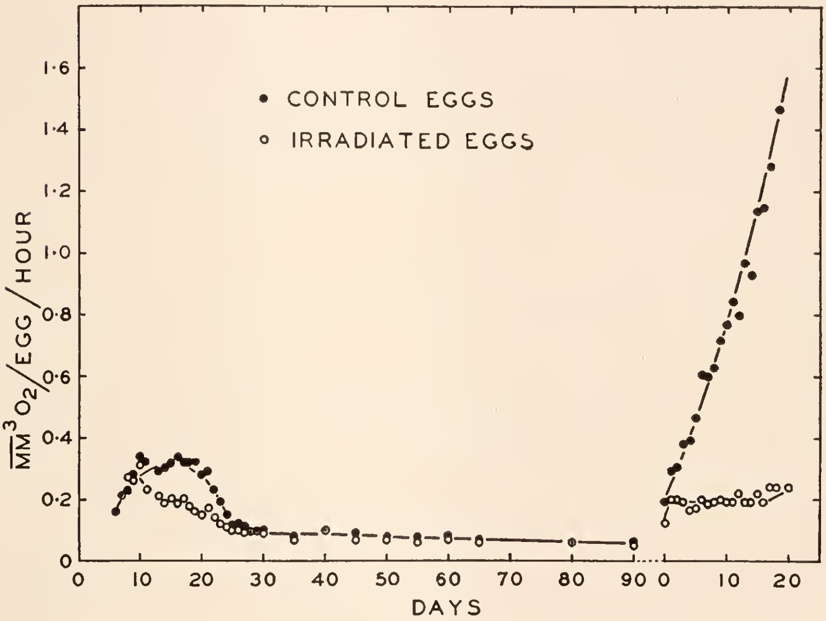

Fig. 1. Shows the rate of oxygen consumption per egg in cubic millimeters per hour. Ordinate, rate of oxygen uptake; abscissa, time in days at 25° C. since laying followed by the time in days at 25° C. after termination of the diapause by exposure to 5° C. for 3 months. ●, control eggs; ○, irradiated eggs. Each point is an average obtained from 3 manometers over a 2- to 3-hour period. Twelve thousand eggs were used in these determinations. Other lots used gave similar results.

yolk or serosa cells. No evidence of the presence of an embryo in irradiated eggs can be found. Further data concerning morphological changes due to x-irradiation are to be discussed in a subsequent communication from this laboratory by Dr. Titus Evans.

The O_2 consumption curves for both irradiated and control eggs are shown in Fig. 1. The respiration of the irradiated eggs increased slightly during the first 3 days after irradiation and then slowly decreased up to the time when the control eggs entered diapause. The O_2 consumption rates of both irradiated and control eggs are similar during diapause. When subjected to 5° C. for 3 months, diapause is terminated in both experimental and control eggs since when they are returned to 25° C. increases in their rates of O_2 consumption are noted. By reference to Fig. 1, it can be seen that respiration in control eggs increases markedly during postdiapause up to the time of hatching. Irradiated

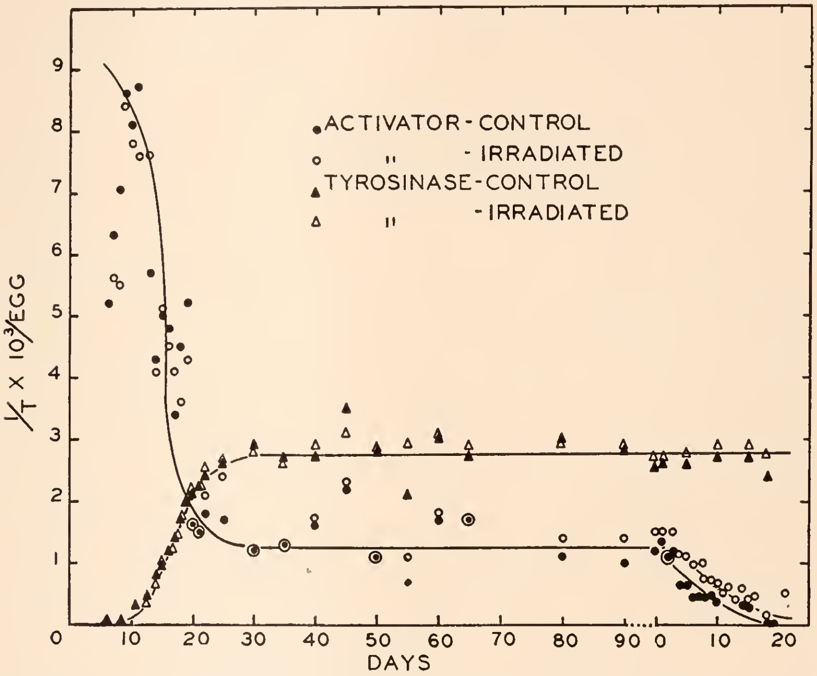

FIG. 2. The formation of protyrosinase and the amount of naturally occurring activator during ontogenesis. The ordinate shows the specific reaction rate per egg. The rate is the reciprocal of the half oxidation period of the substrate multiplied by 10^3. The abscissa is the time in days at 25° C. as described under Fig. 1. ▲, protyrosinase in control eggs; △, protyrosinase in irradiated eggs; ●, activator in control eggs; ○, activator in irradiated eggs. Twelve thousand eggs were used in these determinations.

eggs, on the other hand, increase in O_2 consumption rates for a period of 3 days and reach a maximum at which they remain throughout the course of the experiment.

The growth of the enzyme, protyrosinase, in both control and irradiated eggs is given by curves in Fig. 2. An examination of these shows no difference in the enzyme production in the two types of eggs and these results are a direct confirmation of those described by Ray (1938). Changes in potency of the naturally occurring activator of protyrosinase are also given in Fig. 2. It will also be noted that no significant differences in the type of curve for irradiated and control eggs are apparent. The general shapes of the curve for natural activator are similar to those previously reported (Bodine, Ray, Allen and Carlson, 1939). After the termination of diapause, it will be observed that the decline in potency of the activator in the irradiated eggs is less than that of the control eggs. No explanation of this difference is at hand.

Discussion

It may be well to review briefly the early stages in the development of grasshopper eggs at 25° C. to better appreciate the significance of the results of the present experiments. At the end of the first day after laying, cleavage has progressed and the resulting nuclei are found scattered in the yolk with a few in the peripheral protoplasm. On the second day there are many nuclei, some in the yolk, some in the peripheral layer. During the third and fourth day, the germ band forms and by the fifth day, the embryo is large enough to dissect from the egg (Slifer and King, 1934). On the sixth day, the cells at the periphery (serosa) are secreting the cuticle. The extra-embryonic cells cease mitotic activity after the fourth or fifth day of development and after the seventh day there is no significant change in the character of the yolk and serosa cells (Evans, 1940). The marked resistance to x-irradiation of the serosa and yolk cells permits the destruction of the embryo tissue without damaging either yolk or serosa (Evans, 1936; Ray, 1938). The physiological processes which are evidenced in the measurements made on irradiated eggs are, therefore, most likely accomplished by the yolk and serosa cells. Preliminary experiments show that the respiratory quotient is not markedly changed in the irradiated eggs though the oxygen uptake during the period of active development is somewhat less than that of the control. Bodine and Boell (1936) have shown the relationship between respiration of embryo and whole egg during development and it seems interesting to note that in the present experiments, the periods where the O_2 uptake of the control eggs exceeds that of the irradiated eggs corresponds to the times when the embryo respiration is the major part of the total respiration. They also noted that the initial increase in oxygen uptake when diapause or the block to normal development and cell activity is removed is for the most part due to yolk cells and extra-embryonic material. A rise in oxygen consumption of irradiated eggs (without embryos) occurs at this time, and in fact, it is the period in which the total postdiapause increase in respiration occurs.

These investigations, then, on the rate of oxygen consumption, an enzyme formation (presumably a protein) and the decrease in potency of the activator of this enzyme (a lipoidal constituent) seem to show that definite metabolic processes occur in the yolk and serosa cells even in the absence of an embryo and that these cells may play an important part in the general metabolism of the whole egg. Evidence from the rate of respiration (Fig. 1) and from the decrease in potency of the activator (Fig. 2) seems to demonstrate that these cells in the egg in the absence of the formed embryo appear to become blocked during development and exhibit the general characteristics found in the normal development of the grasshopper egg, i.e. a prediapause, diapause and postdiapause periods.

Further investigations in the morphology and physiology of these eggs to bring out fundamental relationships seem desirable.

Summary and Conclusions

1. The embryos in the egg of the grasshopper (*Melanoplus differentialis*) can be totally destroyed by an x-irradiation of 1000 r on the sixth day of development.

2. Eggs deprived of an embryo in such a manner have been studied in regard to their oxygen consumption, the formation of protyrosinase, and the amount of natural activator of this enzyme.

3. The respiration of the irradiated eggs differs from that of the controls during the active stages of development. The oxygen uptake of the irradiated eggs gradually falls during prediapause until it reaches the diapause level. An increase in the rate of respiration of these eggs occurs during the first 3 days of postdiapause.

4. The enzyme protyrosinase is formed in the same manner in both experimental and control eggs.

5. The potency of natural activator in irradiated eggs does not differ from that of controls until postdiapause when the decrease in potency is slightly less in experimental eggs.

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THE DISTRIBUTION OF ELECTROLYTES IN PHASCOLOSOMA MUSCLE

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In recent years considerable attention has been paid to the distribution of electrolytes in tissues, particularly muscle and nerve. It has been recognized, of course, that the total electrolyte content of such a tissue as muscle must be distributed between at least two phases, one being roughly the cellular phase, the other extracellular.

That protoplasm is a solution of electrolytes, among other things, has been amply demonstrated by observations on conductivity. Gross analysis of masses of cells and tissues shows that these electrolytes must be largely of a type with inorganic cations and organic anions (see Fenn, 1936). Many of the organic substances in cells have been described, but until recently little attention has been paid to the distribution of the inorganic constituents.

The general principle that, in many tissues composed of highly differentiated cells, chloride does not penetrate into the cells (Fenn, *loc. cit.* See also Amberson et al, 1938) is very useful, since it allows calculations to be made of the approximate amounts of other inorganic constituents present in the extracellular space and hence, cellular concentrations, if the total concentration in the tissue is known. The method of study is very simple, involving soaking the tissues in chosen solutions and then analyzing them for chloride and the other elements in question. The data then are treated on the assumptions that all electrolytes of the external medium are in simple equilibrium with the extracellular space (chloride space) and that the total amounts of metallic elements found in the ash may be regarded as a source of positively charged ions, either within or without the cells.

A recent study (Steinbach, 1940) dealing with chloride, sodium, potassium and calcium in *Thyone* muscle has been reported. The results given in the present paper are concerned with similar experiments on another invertebrate smooth muscle, the retractor muscle of the marine annelid *Phascolosoma*.

The structure of these muscles has been described by Olson (1940).

METHODS

The methods used were identical with those reported previously (Steinbach, 1940). The muscles were excised with as little injury as possible and immersed in fifty to one hundred times their weight of solution. Solutions used were either normal sea water, isotonic salt solutions, isotonic buffered sucrose solutions or mixtures of these as indicated. All solutions had a pH of 8.0 to 8.5. The experiments were carried out at room temperature.

RESULTS

Phascolosoma muscles swell when transferred to sea water and their composition changes accordingly. Table I gives average figures for Na, Cl, K and Ca contents of muscles freshly removed from the body and muscles that have been soaked in sea water for several hours. Figures are also included in the table showing the concentrations of these elements in body fluid of *Phascolosoma* and in Woods Hole sea water.

TABLE I

Analysis of *Phascolosoma* muscle, fresh (column 1) and treated with sea water for several hours (column 2). Column 3 gives the analysis of *Phascolosoma* body fluid and column 4 of Woods Hole sea water.* Figures in brackets give number of analyses averaged. Concentrations in milliequivalents per hundred grams.

Element	(1) Fresh	(2) Soaked	(3) Body fluid	(4) Sea water
Na	12.2 [3]	14.9 [6]	37.8 [2]	44
K	10.6 [4]	9.0 [2]	3.8 [2]	0.9
Ca	0.85 [4]	1.14 [4]	2.1 [3]	1.8
Cl	9.1 [8]	16.0 [9]	43.0 [2]	51
Relative weight	100	119		
Dry weight %	22		9	
Ash weight %	2.1			

* Sea water is not constant in composition from year to year. The figure for chloride, for example, is from data of 1938. In 1939 the chloride was 2 to 3 per cent lower.

The change in chloride of the muscles can be almost entirely accounted for by assuming that the change of weight represents an increase in the extracellular space. Sodium is present in fresh muscle slightly in excess of chloride. In soaked muscles the situation is changed and most of the chloride can be accounted for as sodium chloride. A little potassium is lost on soaking and calcium increases slightly.

The main purpose of this work was to follow the loss or gain of cellular electrolytes, using the chloride space (ratio of Cl inside to Cl outside) as a measure of the extracellular space of the whole tissue. In order to do this, it was first essential to show that chloride in the tissue bore a simple linear relationship to the chloride concentration of the medium. Muscles were soaked in various dilutions of sea water with isotonic sucrose solutions and then analyzed. A few experiments were also done in which normal sea water was diluted with an artificial sea water (Allen's formula) made up with nitrates instead of chlorides. The results (Fig. 1) show clearly that chloride in the muscle does bear a simple linear relationship to chloride of the medium and hence the assumption of an extracellular chloride space is probably correct. On this basis, about 30 per cent of the whole tissue is extracellular space. Determinations of sodium were made on muscles similarly treated and since sodium appears to be extracellular in soaked muscles the results are included in Fig. 1.

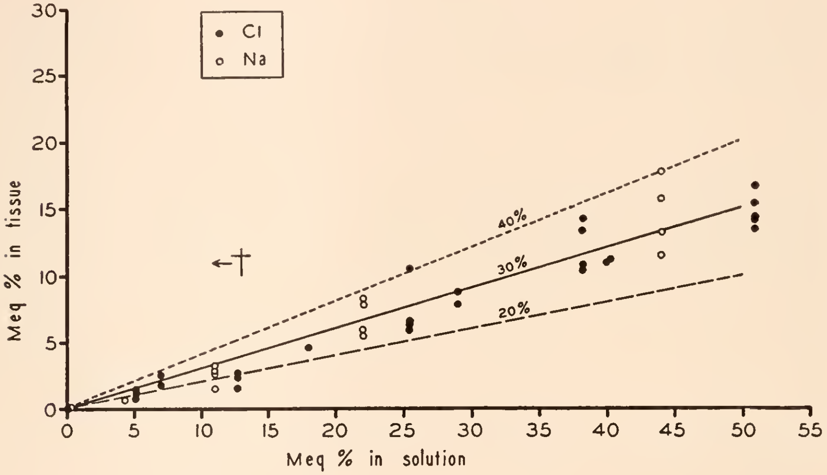

FIG. 1. Chloride (●) and sodium (○) concentrations in *Phascolosoma* muscles plotted against the same elements in solution. The concentrations in the solutions were altered by diluting normal sea water with isotonic buffered sucrose solutions. Tissues soaked 2 to 4 hours before analysis. The cross indicates the minimal salt concentration for maintaining irritability (response to electrical stimulation). All concentrations in milliequivalents per hundred grams solution or final wet weight of tissue. Straight lines represent calculated values for chloride space as indicated. Each point is a single determination on several muscles.

The behavior of potassium in the muscles was studied by immersing muscles in solutions of varying potassium content made by diluting either an artificial potassium-free sea water (Allen's formula) or normal sea water with isotonic KCl solution. For each solution being tested, two batches of muscles were used, the one to be analyzed for potassium, the other for chloride. Initial and final weights were recorded. The results are reported in terms of milliequivalents per hundred grams (meq per cent) final wet weight of tissue. Figure 2 summarizes the results. Potassium is lost from the tissue to K-free

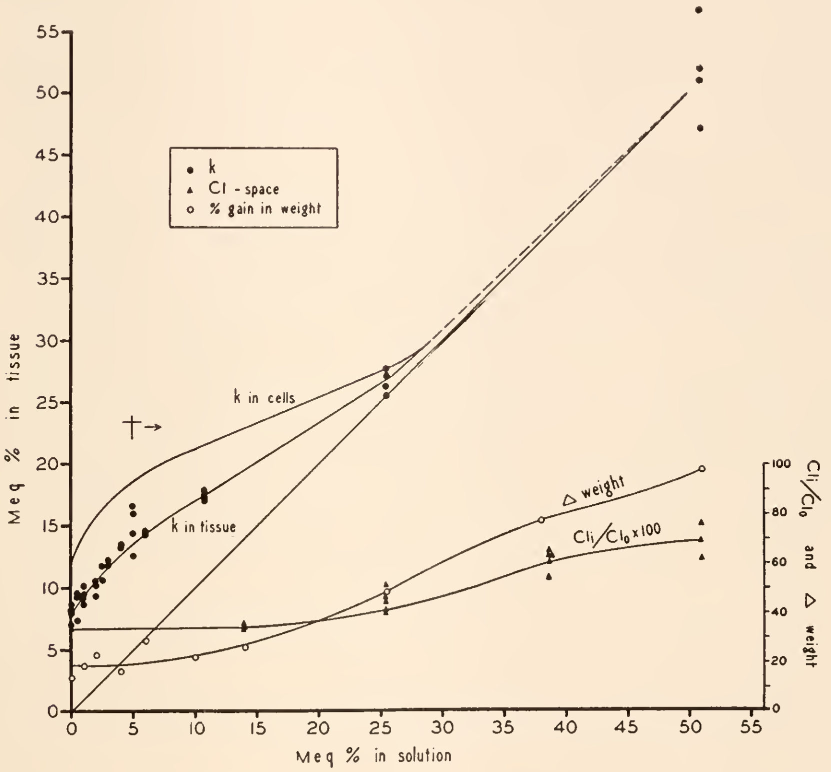

FIG. 2. Potassium concentrations in *Phascolosoma* muscles (●), weight changes (○) and chloride space (▲) plotted against potassium concentrations in the external medium. Tissues soaked 6 to 8 hours. Solutions made up by diluting sea water with potassium chloride solutions. Cross indicates highest potassium concentration at which irritability is maintained. The curve for potassium in the cells represents potassium concentrations calculated as outlined in Table II. Concentrations in milliequivalents per hundred grams solution or final wet weight of tissue.

solutions and is gained in increasing amounts as the potassium content of the medium is increased. Potassium is first concentrated in the tissue but the amount gained for each increment in potassium of the medium becomes less with higher concentrations until, when the solution is about 0.25 M in potassium, tissue and solution have nearly equal concentrations. Further increases in potassium of the medium do not appear to disturb this last relationship. The potassium concentration in the cells was estimated by subtracting the potassium present in the chloride space ($Cl_i/Cl_o \times$ K-conc. of solution) from the total potassium and dividing the results by the cell space ($1 - Cl_i/Cl_o$). Values

TABLE II

Calculated figures for potassium concentrations in the cells (column 5) and cell volume (column 6). Calculations performed using figures read from the smoothed-out curves drawn through the experimental points of Fig. 2. Concentrations in milliequivalents per hundred grams.

$$\text{Relative weight (column 2)} = \frac{\text{final weight}}{\text{initial weight}} \cdot \quad Cl_i/Cl_0 = \frac{\text{chloride in tissue}}{\text{chloride in solution}} \cdot$$

K concentration in cells (column 5) calculated by subtracting product $Cl_i/Cl_0 \times$ K in solution from K concentration of tissue and dividing by cell space ($1 - Cl_i/Cl_0$). Cell volume calculated by multiplying relative weight by cell space. Average chloride of all solutions assumed to be 52 meq per cent.

(1) Conc. K solution (fresh muscle)	(2) Relative weight tissue	(3) Conc. K tissue	(4) Cl_i/Cl_0	(5) Conc. K cells	(6) Cell volume
	100	10	0.17	11.8	83
0	119	8	0.33	11.9	80
5	120	14	0.33	18.5	80
10	123	17	0.33	22.0	82
15	128	20	0.35	22.8	83
20	133	23	0.37	24.7	84
25	139	26	0.42	26.7	81
35	171	35	0.57	34.8	73
50	199	50	0.67	50.0	66

for these calculations were read from the smoothed-out curves of Fig. 2 and the results are plotted in Fig. 2 (upper curve). These results merely indicate that the concentration of cellular potassium is considerably higher than the potassium concentration of the medium at first, the difference decreasing with increasing concentrations until when the solution is about 0.25 M in potassium the two phases are equally concentrated. Since the cells contain most of the dry substance, this means that, on the basis of water content, the cells always contain a higher concentration of potassium than is found in the external medium.

The calculations of cellular potassium concentrations are calculations based on the assumption that chloride does not penetrate the living cells. That is, cellular potassium concentration figures really represent the amount of potassium present in excess of chloride and presumably occupying space not penetrated by chloride. The mechanism for partitioning the elements in this fashion may or may not be the cell membrane but whatever it is, it has the interesting result that "cell space" remains almost constant in spite of the fact that the muscle swells to almost double its original size. This fact is brought out clearly by the figures given in Table II. These figures are calculated from data read from the smoothed-out curves of Fig. 2. Cell size (volume of muscle not containing chloride) is calculated by multiplying the final relative weight (initial weight = 100) by the cell space $(1 - Cl_i/Cl_o)$. From the figures shown it is apparent that these muscles can increase in weight by as much as 40 per cent and more than double the chloride space without any appreciable change in the calculated cell volume. It would seem rather doubtful that this actually means a constant cell size. A more liberal and probably more correct interpretation of the results would be that the protoplasm, due to its whole chemical and physical structure, represents non-solvent space as far as chloride is concerned. Permeability might be a factor in this but modified Donnan equilibria or other chemical equilibria must also be important. This interpretation probably holds also for *Thyone* muscles but is not needed so obviously since those tissues do not swell to any great extent in solutions of high potassium concentration.

The calcium balance in the tissue was studied by methods similar to those used for potassium, except that dilutions were made with calcium chloride solutions. The results of the studies are shown in Fig. 3. There is a nearly linear relationship between the calcium of the muscle and calcium of the solutions except that a small residue remains in the muscle even after prolonged soaking in calcium-free solutions. The slope of the line drawn through the experimental points is greater than that of Fig. 1, showing that some calcium is probably entering the cells. On the other hand, the results also show that calcium is never as concentrated in the cells as in the external medium so long as the outside concentration is above that of normal sea water.

As the calcium of the medium is increased, swelling of the tissues becomes less and the chloride space increases. This may indicate that excess calcium actually causes a shrinkage of the cells but it might also show that some cells are being destroyed. Calculations show that not only is total cellular calcium lower in tissues in high-calcium solutions,

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but the concentration of calcium in the cells is also lower. This is similar to the situation found in *Thyone* (Steinbach, 1940) and shows that there must be some change in the cells under the influence of high external calcium so that less calcium can enter or stick to them.

Calculations similar to those made for potassium show that as the calcium of the medium increases the cell size decreases. This is apparent from inspection of Fig. 3. The total weight of the tissue is less when the calcium is high outside and the chloride space increases; therefore there must be a decrease in cell (non-chloride) space. This may mean either shrinkage or destruction of cells. These results do not differentiate between the two possible changes.

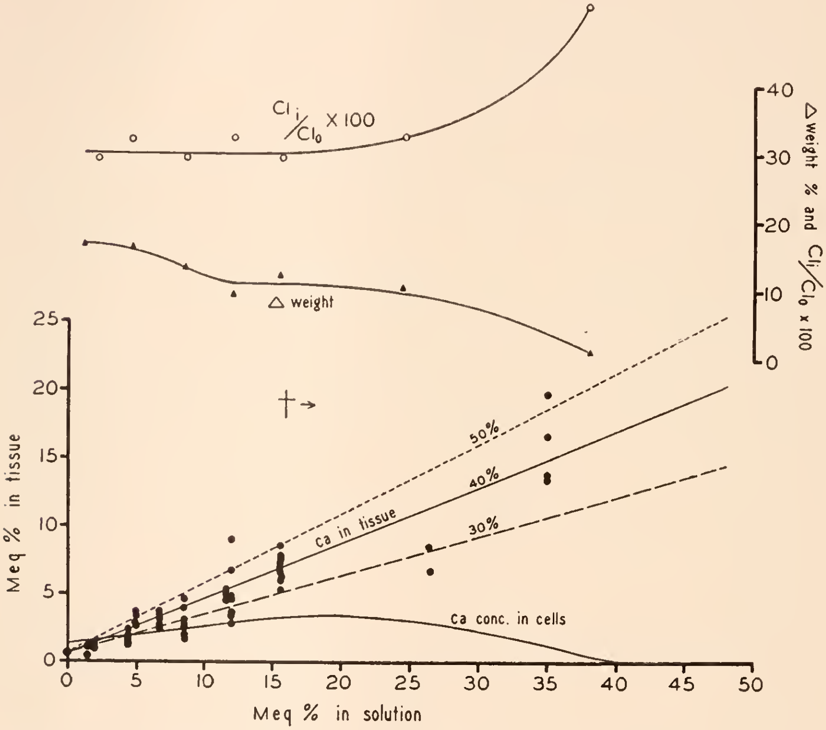

FIG. 3. Changes in *Phascolosoma* muscles as the calcium concentration of the medium is altered. Symbols, concentrations and methods of calculation as in Fig. 2.

DISCUSSION

The concept of the chloride space of muscle tissue is a useful one. The evidence is good that under normal conditions all of the chloride is extracellular in frog striated muscle, and is quite satisfactory for other vertebrate tissues as well (see Manery and Hastings, 1938). Normal *Thyone* muscle appears to contain chloride only in the extracellular spaces and the present evidence would show that the same holds true in *Phascolosoma*. Both these latter muscles would be classed as smooth muscles; thus another similarity is shown between smooth and striated muscle tissue.

The constancy of the chloride space under normal conditions and the regular way in which it changes with various experimental procedures can be regarded as evidence that there is some definite exclusion of chloride from part of the muscle volume. It is, however, a little difficult to conclude that the cells of *Phascolosoma* muscle change as little in size as is shown in Table II, when the whole muscle doubles in weight. It seems more probable that the partition of chloride is not a definite exclusion by a discrete membrane but depends upon a number of chemical and physical factors so balanced that there is a certain volume of tissue that is, for want of a better term, non-solvent space with respect to chloride. This non-solvent space for chloride probably is nearly equivalent to cell space in normal tissue. But in greatly swollen tissue this may or may not be true. Some start has been made toward a histological study of muscles swollen in KCl and the results, while inconclusive, would indicate that the cells do not stay the same size under all these conditions. It probably is best, then, to relate chloride in swollen muscles to the organic content of the protoplasm rather than to size of cells. On this basis the calculated cell sizes given in Table II would then show that the organic (dry weight) constituents of the cells were still present, occupying space and influencing chemical equilibria as they do in normal cells.

On this basis, the concentration of potassium in such a manner that there is always more potassium inside cells than outside, figured per unit weight of water, probably represents potassium entering into some special equilibrium with the protoplasmic constituents rather than being locked within a membrane. The two mechanisms, of course, have the same effect, but a membrane explaining the results reported here would have to possess most remarkable properties.

Several problems arise in connection with the potassium studies. Potassium enters the muscle cells so that there is always more potassium inside than outside. There can be little sodium within the cells and other inorganic cations are present in low concentration. Since chloride does not enter, there is then the problem of accounting for the mode of existence of the potassium. The cells are obviously in osmotic equilibrium with their normal environment and yet the internal potassium content can be doubled without a change in calculated cell size. Potassium then enters where there was no potassium before, no swelling occurs and there is no common cation exchanged or anion entering. The only possible solution would seem to be the creation of new electrolytes, probably organic acids, from non-electrolytes previously active only in maintaining osmotic balance. There is at present no clue as to the nature of this process or how it might take place.

The calcium balance of the cells presents as much of a problem as the potassium balance but here the mechanisms concerned allow a partial penetration of calcium, not a concentration of the element. Except with very low external calcium, there is always less of the element in the tissue than in the medium. Furthermore, the changes in calcium content of the cells indicate that the ability to take up calcium from the environment is lowered by treatment with excess calcium in the medium. In view of the demonstrated importance of calcium in surface phenomena (see Heilbrunn, 1937), it seems probable that calcium never penetrates completely within the cell but is stopped by some surface equilibrium condition.

SUMMARY

An analysis is presented of the major inorganic constituents of *Phascolosoma* muscle and body fluid. As is usually found in muscle tissue, potassium is more concentrated in the tissue than in the body fluid or in sea water. Calcium is in about the same concentration while sodium and chloride are in the tissue in lower concentrations than they exist in the external medium.

Sodium and chloride appear to be extracellular in these muscles, most of the potassium and some calcium are intracellular. Parallel analyses of muscles for different elements show that the cells can take up considerably more potassium than they normally contain without an increase in chloride. Some calcium can also be taken up by the cells.

These findings are discussed briefly in connection with their bearing on problems of permeability to electrolytes of muscle cells.

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EFFECTS OF MARINE MUD UPON THE AEROBIC DECOMPOSITION OF PLANKTON MATERIALS[1]

CHARLES E. RENN

(From the Woods Hole Oceanographic Institution[2] and the Harvard University Graduate School of Engineering[3])

A number of recent investigations of bacterial activity in natural fresh and salt waters, in soils, and in muds strongly suggest that the rates of bacterial decomposition in these sites may be modified by finely divided and colloidal inorganic materials.

It is noted that marine bacteria are carried from suspension by settling marine and salt lake muds. To an undetermined degree encapsulated clumps, slime masses, and bacterial chains are mechanically occluded in the precipitating material, but the ratio of cells removed bears a relation to the electrical charges borne by the muds and bacteria characteristic of physical adsorption (3, 4, 5). Terrestrial soils also carry down adhering bacteria from suspensions. The degree of concentration may be varied by altering the charge on the soil particles (3).

Many marine and fresh water bacteria are sessile in habit, and it has been observed that the amount of bacterial substance in the attached films that these organisms form greatly exceeds the concentration in the surrounding water (6, 7). When the ratio of surface/volume in stored sea water is increased, the bacterial numbers rise and various physiological processes are stimulated. It has been suggested that the higher biological oxygen consumption associated with the increase in surface may be related to the adsorption and concentration of soluble organic materials and bacterial enzymes upon the enclosing surfaces (8).

Soluble and finely divided organic materials are themselves adsorbed on fresh water muds. River silts carry organic matter from muddy water to the bottom as they settle (2). From comparative studies of the biological oxygen demand characteristics, it appears that the organic matter in the silts breaks down at a much lower rate than that of the water from which they settled.

The experiments reported in this paper are attempts to discover if marine muds affect the rates at which plankton materials undergo aerobic decomposition in the superficial layers of the sea bottom. Studies on the breakdown of sewage sludges indicate that aerobic conditions may prevail in the upper centimeter of the active mass, and it is probable that this also applies to the more flocculent surface muds receiving freshly settled debris from the sea (1).

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[1] The author is indebted to the Elizabeth Thompson Science Fund for a grant of money which made possible the construction of the special respirometer apparatus and supplementary equipment used in this investigation.

[2] Contribution No. 257 from the Woods Hole Oceanographic Institution.

[3] Contribution from the Harvard Graduate School of Engineering No. 277.

Surveys of the distribution of organic matter and bacteria in marine muds indicate that biological changes go on relatively slowly in the sea bottom. It was thought, initially, that the adsorption of organic matter on the materials of the mud might be a significant factor in limiting bacterial activity.

EXPERIMENTS ON THE ADSORPTION OF PLANKTON MATERIALS BY MARINE MUD

Materials

The mud used in these experiments was prepared from a series of short cores, 4 to 6 inches deep, taken from a station in the Gulf of Maine (N. 42° 25′, W. 70° 35′) at a depth of 100 meters. It had been stored for a year in the laboratory at room temperatures. A week before the work began the material was mixed with "aged" sea water and repeatedly suspended to remove heavy sand, fine gravel, shell particles, and other large fragments. A final suspension was made and aerated gently for three days. This was allowed to set—the heavy fraction separating within thirty seconds was discarded, and the remainder drawn off for use. The gross analysis of this mud was as follows:

Loss of weight on drying at 105° C. (corrected for salt content)	71.0 per cent
Loss of weight on ignition (on dry weight, salt-free basis)	11.5 per cent
Total nitrogen (on dry weight, salt-free basis)	0.20 per cent
Content of nitrogen per ml. wet mud as used in the experiments	0.70 mgm./ml.

Fresh plankton suspensions were prepared for each experiment from tows taken in Vineyard Sound (collected with No. 20 silk net, chilled, and prepared within four hours). This material consisted predominantly of species of *Rhizosolenia*. The tow was ground and filtered by suction through washed No. 52 Whatman filter paper. Nitrogen content of the extract was determined by micro-Kjeldahl, and convenient sea-water dilutions were prepared from the concentrated stock.

Determination of Adsorption

Varying concentrations of mud and plankton extract were stirred together on a gently rocking shaker for different intervals of time. Samples were withdrawn and nitrogen analyses made on aliquots of the mixture. Fifty-milliliter quantities of the mixture were placed in a clinical centrifuge and whirled until clear. Separate nitrogen determinations were made upon the mixture, upon the supernatant, and upon the centrifugate. The adsorption of plankton extract on mud in various mixtures under these conditions is demonstrated in the data given in Table I.

TABLE I

Adsorption of Nitrogenous Materials by Marine Mud

A. Plankton Extract

Concentration of N in extract	0.60 mg./ml.
Concentration of N in mud	0.70 mg./ml.

Ratio of mud to extract	N in supernatant after centrifuging (three-hour mixing)	N adsorbed by mud in three hours	N in supernatant after centrifuging (twelve-hour mixing)	N adsorbed by mud in twelve hours
	mg./ml.	*per cent*	*mg./ml.*	*per cent*
1 : 5	0.36	40	0.34	43
1 : 2.5			0.31	49
1 : 1	0.23	61	0.20	67
2 : 1	0.12	80	0.11	82

B. Peptone Solution

Concentration of N in solution	0.55 mg./ml.
Concentration of N in mud	0.70 mg./ml.

Ratio of mud to solution	N in supernatant after centrifuging (twelve-hour mixing)			N adsorbed by mud in twelve hours
	mg./ml.			*per cent*
1 : 25	0.42			23
1 : 2	0.13			77

It is evident from this that the nitrogen-bearing fraction of plankton extracts are strongly adsorbed on marine mud. The concentrations involved in these experiments are several thousand times as high as those occurring in sea water but fall within the ranges that occur in marine muds.

Soluble materials such as plankton extract and peptone are readily adsorbed at room temperatures, but bacterial digests of the same material are not removed to a comparable degree. In an experiment with a dense suspension of mixed bacteria grown in plankton extract, only 10 per cent of the nitrogen was adsorbed from suspension by mud stirred for 12 hours and centrifuged under conditions comparable to those applying in the other experiments. Microscopic examination of the mud thrown down showed the bacteria moving freely through spaces between particles—the centrifugate remained cloudy with motile bacteria.

Experiments on the Effect of Mud on Rates of Aerobic Decomposition

Breakdown of Particulate Plankton

Previous experiments on the rates of decomposition of organic matter in marine muds have been quantitatively unsatisfactory because of the technical limitations of the standard biological oxygen demand method. In these experiments the difficulties were eliminated by using a series of respirometers. The apparatus was designed by the author for the direct measurement of oxygen consumption of such organic matter rich systems as grossly polluted waters, sewage sludges, and manured soils. A description of their design and applications will be published in the near future.

In the first experiment the respirometers were filled with mixtures of mud and finely ground, unfiltered plankton. Duplicate respirometers were used for each mixture and filled as follows:

Set A—4 units mud in 200 ml. aged sea water
Set B—2 units mud, 1 unit plankton in 200 ml. aged sea water
Set C—1 unit mud, 2 units plankton in 200 ml. aged sea water
Set D—4 units plankton in 200 ml. aged sea water

Carbon analyses were not available for the plankton material, but satisfactory approximations may be made from the nitrogen determinations. Assuming a C/N ratio of 10:1 for the organic matter of the mud, the organic carbon value of the unit of mud is about 3.5 mgm. If a C/N ratio of 8:1 is taken for the plankton, the value of the plankton unit in terms of organic carbon is 2.4 mgm.

During the five-day term of this experiment the temperature was 23° C. ± 0.5° C.

The oxygen consumption of the various mixtures is plotted against time in Fig. 1.

Greatest irregularities occur in the duplicate samples of mud—possibly through the accidental inclusion of organic particles. It can be assumed that the oxygen consumed in this interval was utilized in the biological oxidation of the organic carbon in the plankton material. On this basis a little over 50 per cent of the plankton carbon was oxidized during the five days of activity. The organic matter of the mud is more stable—in the same interval about 7 per cent of this carbon underwent oxidation.

In the mud and plankton mixtures the amounts of organic carbon oxidized to carbon dioxide are not significantly different at any time from the values that would be estimated from the consumptions of the separate components. There is a slight increase in the proportion of carbon oxidized in the mixtures, but this effect is most pronounced in the lower concentration of plankton and may be related to the dilution effect observed in the third experiment.

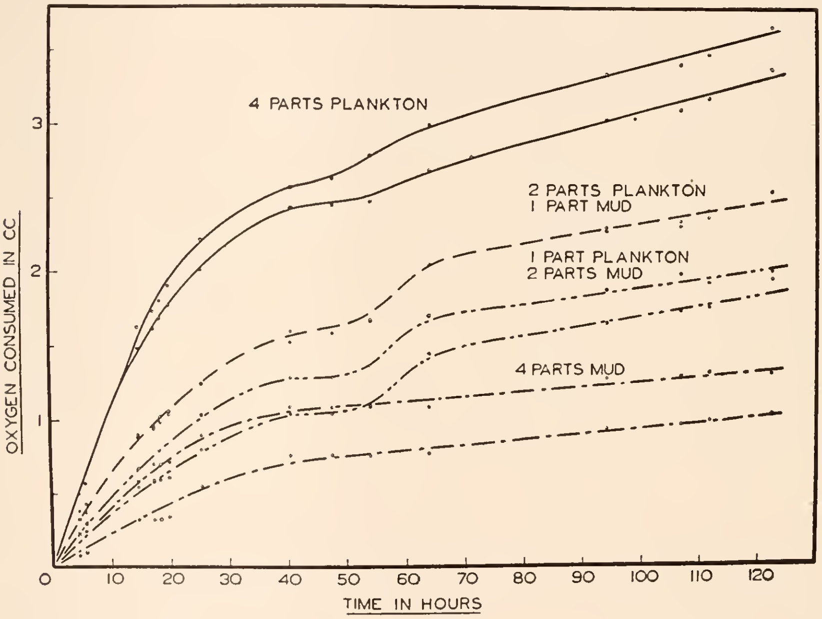

FIG. 1. Biological oxygen consumption in sea water suspensions of mud (lower set of curves), of ground plankton (upper curves), and of mixtures of mud and plankton (intermediate curves).

Breakdown of Plankton Extract

A second experiment was prepared, using plankton extract instead of fine, particulate plankton. Mud, plankton-extract, and sea water were placed in sets of duplicate respirometers as follows:

Set A—4 units mud in 200 ml. aged sea water
Set B—2 units mud, 1 unit plankton, 200 ml. sea water
Set C—1 unit mud, 2 units plankton, 200 ml. sea water
Set D—4 units plankton in 200 ml. sea water

The nitrogen values of the mud and plankton units in the charges were adjusted to equal those of the first experiment. During the five days of activity the temperature ranged about 23.5° C. ± 0.5° C.

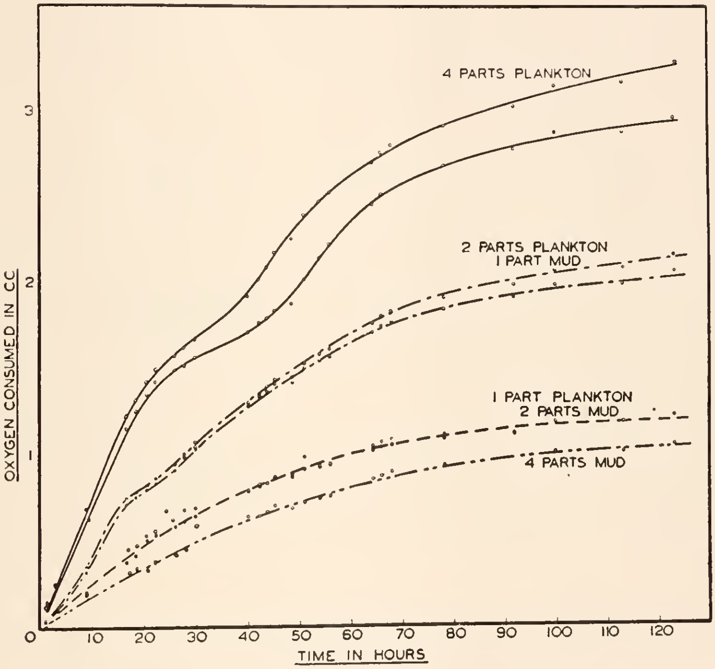

Fig. 2. Biological oxygen consumption of sea water suspensions of mud, plankton extract, and of mixtures of mud and plankton extract.

At the end of the second experiment approximately 45 per cent of the plankton carbon had been oxidized and between 6 and 7 per cent of the organic carbon in the mud. Very slightly more oxygen was consumed in the mixtures than would be calculated, but this difference is not significant when the greater efficiency of breakdown in the more dilute suspensions (Exp. 3) is considered.

Effect of Concentration on Decomposition in Mud

In the final experiment an attempt was made to discover what effect might be expected in rich mixtures of mud and plankton as compared with the relatively dilute suspensions of mud and plankton used in the first two experiments. The respirometers were loaded in the following manner:

Set A—4 units mud (no added sea water)
Set B—2 units plankton extract, 2 units mud
Set C—2 units plankton extract, 2 units mud, 100 ml. aged sea water
Set D—4 units plankton extract (no added sea water)

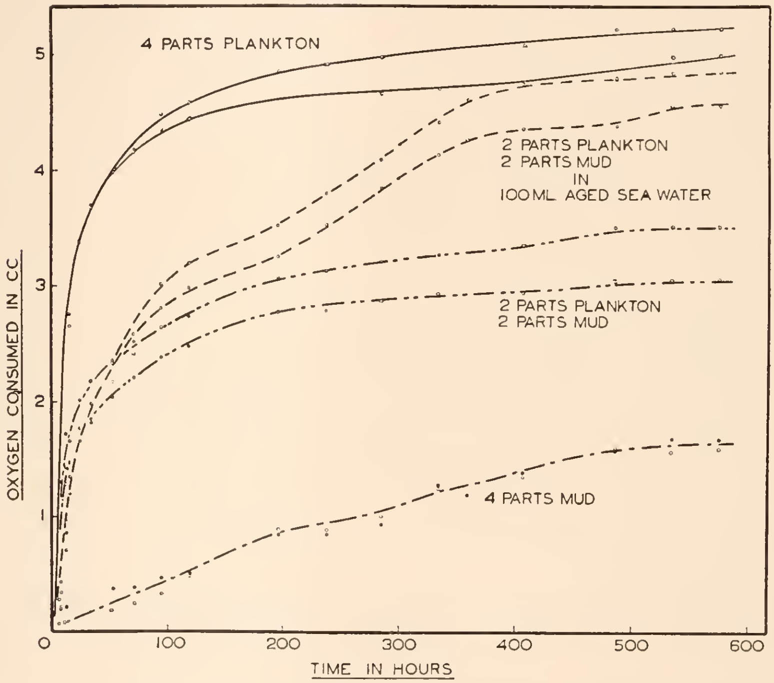

FIG. 3. Biological oxygen consumption concentrated mud, plankton extract, mixtures of plankton extract and mud, and of a sea water suspension of mud and plankton extract.

Although the rate of oxygen uptake had markedly decreased by the end of five days at 23.0° C. ± 0.5° C., the experiment was continued for 24 days.

At the end of this time 75 per cent of the organic carbon in the plankton extract had been oxidized and about 16 per cent of that in the mud. The undiluted mixture of mud and plankton consumed oxygen at a rate practically equal to that anticipated from the rates of consumption in the separate mud and plankton cultures.

The oxygen demand curve of the mud and plankton mixture diluted with 100 ml. of sea water is markedly different, however. It indicates a more rapid consumption of oxygen during the first hundred hours and a prolonged demand during the next three hundred hours. The oxygen consumed at the end of the active period—300 hours—is about 50 per cent greater than that taken up by the concentrated mud and plankton mixture. This effect of dilution is not related to the oxygen consumption of the added, aged sea water. Repeated determinations were made on the water alone and in no case did the oxygen demand of five-day intervals exceed 0.2 ml. per liter.

Discussion

It appears from these experiments that marine muds have little if any direct effect upon the rates at which readily decomposed organic materials that may be mixed with them undergo aerobic decomposition.

The fact that marine muds adsorb nitrogenous organic materials may be significant, however, in determining the normal course of their breakdown. Soluble organic matter released by autolytic or digestive processes may be localized in the mud where anaerobic changes and low temperatures prevail.

It is unlikely that silts perform transport functions in the sea as they do in the slow-flowing parts of silt-laden rivers, but by slowing down the rates of diffusion and maintaining relatively high local concentrations, muds may lower the effective rate of bacterial decomposition in the bottom.

Summary

1. Marine muds strongly adsorb soluble nitrogenous organic materials of plankton origin.

2. Muds exert little if any direct effect upon the rates or efficiency of anaerobic bacterial decomposition of plankton materials.

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THE LOCATION OF FACTORS OF HEAD REGENERATION IN THE EARTHWORM[1]

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INTRODUCTION

Common earthworms, within a certain limited extremely anterior region, can completely regenerate lost segments. At successively more posterior segments, the capacity for regeneration of characteristic head structures diminishes. Within a certain limited approximately middle region of the worm, a " tail " usually develops at a posterior cut surface, and a tail in reversed orientation (heteromorphic) may be produced at an anterior surface, but simple transection is never followed by development of a head. In the posterior region of the worm regeneration of a tail occurs at only the posterior cut surface.

These two questions arise. What determines regeneration? Is it a function of all of the organs and tissues present at the cut surface, or is some one part or group of parts responsible for the occurrence and character of regeneration? The discovery of " organizers " in embryonic development suggests the search for localized agencies determining regeneration.

A little analysis of the problem recognizes three major parts of the animal, in one or more of which the determining agencies may be seated: (1) the body wall, consisting mainly of epidermis (ectoderm) and muscle; (2) the digestive tube, including an internal epithelium and a muscle layer; (3) the central nervous organ. It seems unlikely that vascular organs or connective tissue should be directly concerned.

The central nervous structures have long been suspected of having some peculiar importance in relation to the occurrence and nature of regeneration. This has been investigated by Morgan (1902), Goldfarb (1909, 1914), Siegmund (1928), Avel (1932), Crowell (1937), and Bailey (1930, 1939). Very little has been said about the anterior body wall except that its epidermis may contribute to the regeneration of the new nervous parts. Among those who have taken a stand either for or against the epidermal origin of nervous parts are Hescheler (1898), Rand (1901), Nuzum and Rand (1924), Bailey (1930) and Schwartz

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[1] Presented at the Thirty-sixth Annual Meeting of the American Society of Zoologists, 1938. *Anat. Rec.*, 72: (4) Suppl., p. 85.

(1932). Morgan (1902), Hunt (1919), and Nuzum and Rand (1924) have dealt with the part played by the digestive tube.

The experiments described in the present paper were designed to test body wall, digestive tube, and nerve cord as to their relation to the initiation of regeneration and the kind of structure regenerated. The method consisted in transferring (by transplantation operations) one or two of the three parts in question from the anterior head-regenerating region to the more posterior tail-regenerating region, or in the reverse direction. The general idea was to make all possible combinations of body wall, gut, and nerve cord of head-forming and tail-forming regions of the worm.

MATERIALS AND METHODS

The common earthworm, *Allolobophora caliginosa,* was used in all experiments. Among those used were a few (perhaps six) more highly pigmented than the others. Except for the pigment, however, no difference could be detected.

Previous to operation, the worm was anesthetized by placing in 0.2 per cent chloretone for about ten minutes. It was then laid on a glass plate covered by a wet paper towel. A simple tripod lens, or in difficult cases a binocular dissecting microscope, was used. Ordinary small scissors and forceps, sometimes jeweler's forceps, were adequate. Especially useful were sharpened needles inserted into large match sticks.

After the transplantation, stitches were taken with the use of a small needle (No. 12), and one strand of a fine three-strand silk thread. The worm was then placed in a glass tumbler between layers of wet paper toweling. The tumblers, each covered by a square of glass, were kept in a cold room at 3° C. for three or four days and then moved to a water-table where the temperature was about 7° C. This temperature was maintained for nearly three weeks. Following this, room temperature was considered most favorable for regeneration. At the desired stages, the worms were fixed in Bouin's picro-formol solution, serially cut in sagittal plane at 8 μ, and stained in Ehrlich's hematoxylin and eosin.

EXPERIMENTAL WORK

Following is an outline of the several types of experiments employed:

Series A. Digestive Tube.
- Type 1. Gut and nerve cord of a region behind clitellum removed and pharynx implanted.
- Type 2. Gut and nerve cord of a region behind clitellum replaced respectively by pharynx and anterior nerve cord.

- Type 3. Gut of a region behind clitellum replaced by pharynx; posterior nerve cord left in position.

Series B. Nerve Cord.

- Type 1. Nerve cord of a region behind clitellum replaced by nerve cord from head-forming region.
- Type 2. Nerve cord in head-forming region replaced by nerve cord from tail-forming region.

Series C. Body Wall.

- Type 1. Segments from head-forming region, including gut but lacking the central nervous organs, sewed in normal antero-posterior orientation to anterior surface of a post-clitellar segment; protruding nerve cord of host component pulled into graft.
- Type 2. Anterior body-wall rings from head-forming region slipped over intestine back of clitellum ("sleeve operation"):
  - (*a*) no central nervous organs present within graft;
  - (*b*) anterior nerve cord present within graft;
  - (*c*) posterior cord drawn forward into graft;
    1. "sleeve" placed in normal orientation,
    2. "sleeve" placed in reversed antero-posterior orientation.

The experiments in which rings of post-clitellar body wall were grafted over the pharynx are not included in the above outline. The operations were unsuccessful, due in large part to the stretching undergone by the posterior body-wall rings as they were pulled over the bulky pharynx. The single animal which survived the above operation gave no evidence of the initiation of either head or tail regeneration. A dozen other cases in which the pharynx was replaced by intestine from a post-clitellar region are omitted from the outline because none survived.

The post-clitellar region to which pharynx, anterior nerve cord, or anterior body wall was transferred lies at various levels between the fifth and twentieth segments behind the clitellum. From this same region parts were transferred to the anterior head-forming region of the worm. The work of Crowell (1937) showed that this post-clitellar region is capable of giving rise to a normal tail in a posterior direction, and a heteromorphic tail in an anterior direction. A series of controls set up by simple transection five, ten, and fifteen segments behind the clitellum verified Crowell's findings. This region was selected, therefore, because in normal regeneration it never produces anything except a tail.

The text figures illustrate not only the several types of experiments employed, but also particular cases which are to be considered as representative of the types. The plate figures are from photographs of sections, and should be compared with the diagrams of the results, shown in the text figures. In the case histories such expressions as "segments 4–11" are always to be understood as including the two segments whose numbers are given.

The text figures are diagrams of median longitudinal sections of the earthworm. The arrows indicate the source and new site of transferred parts and also the discarding of parts. The body wall is stippled; the nervous organs are in solid black. In the diagram representing the result of an experiment the wall of the digestive tube is cross-hatched. Omission of segments is designated by the irregular breaks in the diagrams.

Abbreviations:

A. N.c.	anterior nerve cord
C.	clitellum
I. N.c.	implanted nerve cord
Int.	intestine
P.-c.	post-clitellar
Ph.	pharynx
P. N.c.	posterior nerve cord
R. N.c.	regenerated nerve cord

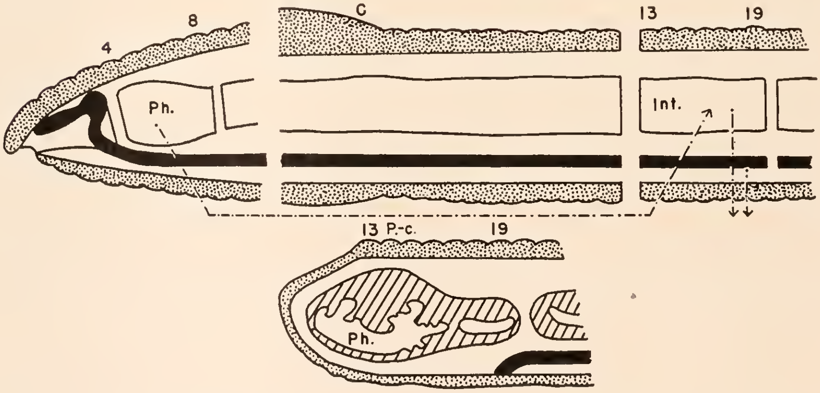

FIG. 1. The pharynx and oesophagus of segments 4–8 replace the intestine and nerve cord of post-clitellar segments 13–19. Result, shown below, is negative.

Series A. Digestive Tube

Type 1. Gut and nerve cord of a region behind clitellum removed and pharynx implanted.

Case 77 (Figs. 1 and 12).—The intestine and nerve cord of post-clitellar segments 13–19 were removed. To this position were transferred, in normal orientation, the pharynx and oesophagus of segments

4–8. The injured head region was then removed. After healing of the posterior incision through which the implant had been inserted, transection was made between the twelfth and thirteenth post-clitellar segments. The worm was fixed at the end of three and one-half months.

Although well oriented with reference to adjacent parts, the pharynx does not open to the outside (Figs. 1 and 12). The nerve cord, turned toward the ventral body wall, reveals little or no growth activity at its anterior end.

In most of the other cases of this type the transection following the healing of the longitudinal incision was made in such a way as to remove the anterior third of the pharynx. It was felt that establishing a freshly cut surface of the pharynx as well as of the body wall would be more conducive to regeneration. In all cases, however, the wound-surface healed over smoothly and no regeneration occurred.

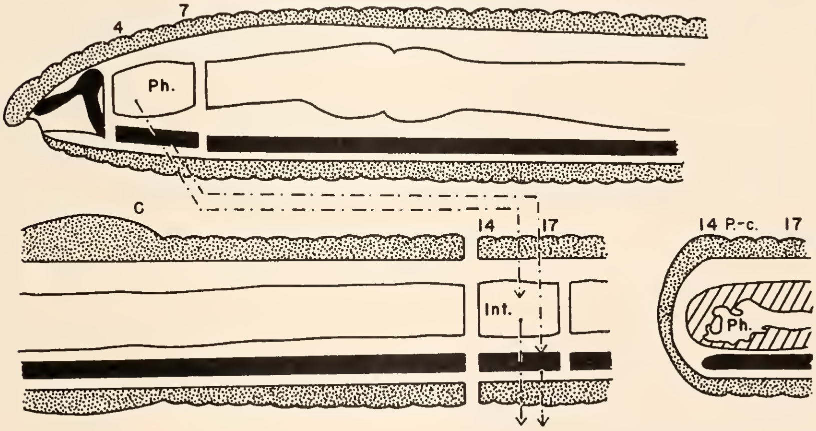

FIG. 2. The pharynx, oesophagus, and nerve cord of segments 4–7 replace the intestine and nerve cord of post-clitellar segments 14–17. Result, negative.

Type 2. Gut and nerve cord of a region behind clitellum replaced by pharynx and anterior nerve cord.

Case 11 (Figs. 2 and 22).—The intestine and nerve cord of post-clitellar segments 14–17 were removed. In their place were implanted, in normal orientation, the pharynx and a portion of the oesophagus together with the nerve cord from segments 4–7. After healing, transection was made at such a level as to remove a small part of the implanted pharynx. The remainder protruded to some extent. At the end of a month, the worm was fixed.

Except for a little proliferation at the forward end of the implanted cord, the result is negative (Figs. 2 and 22). Although communication between the oesophagus and the intestine was established, no anterior perforation to the outside occurred; the cut surface healed over smoothly.

An exceptional case (No. 84, Figs. 20 and 21) of this same type requires special attention. The implant, consisting of pharynx, oesophagus and nerve cord from segments 4–8, replaced the intestine and nerve cord of post-clitellar segments 13–23. After healing, transection was made immediately anterior to the implant. At the end of two and one-half months the worm was fixed. The sections (Figs. 20 and 21) show a well-formed brain, and commissures extending down to the nerve cord. Communication between the oesophagus and the intestine was established, but no anterior opening from the pharynx to the outside. The outstanding feature of the case is the close relationship between the nervous tissue and the pharynx epithelium (Fig. 21).

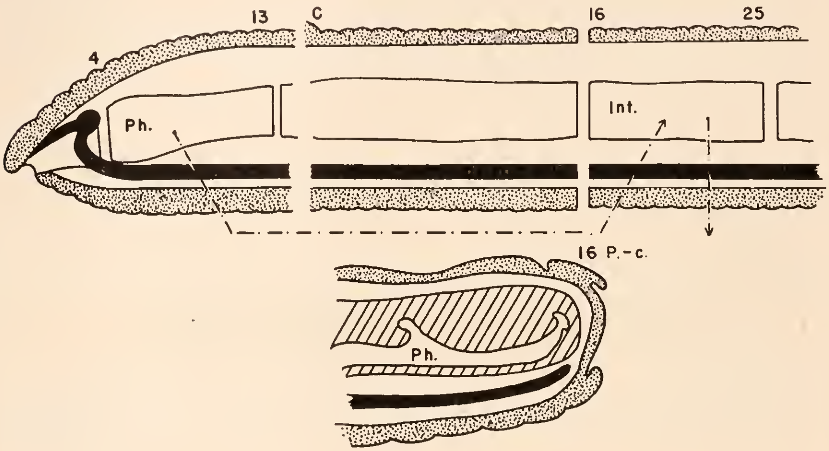

Fig. 3. With posterior nerve cord left in position, the intestine of post-clitellar segments 16–25 is replaced by pharynx and oesophagus of segments 4–13. Result, negative. The antero-posterior orientation of the lower part of the diagram is reversed to correspond to that of Fig. 16.

Type 3. Gut of a region behind clitellum replaced by pharynx; posterior nerve cord left in position.

Case 68 (*Figs. 3 and 16*).—The pharynx and oesophagus from segments 4–13 replaced, in normal orientation, the intestine of post-clitellar segments 16–25. The posterior nerve cord was left in position.

After healing of the longitudinal incision, transection was made between the fifteenth and sixteenth post-clitellar segments. Two and one-half months later the worm was fixed.

The sections reveal little cellular activity other than wound closure and healing. Certain sections show some proliferation of cells from the anterior end of the nerve cord, but give little evidence that a brain might have regenerated.

Series B. Nerve Cord

Type 1. Nerve cord of a region behind clitellum replaced by nerve cord from head-forming region.

Case 60 (*Figs. 4 and 23*).—The nerve cord of post-clitellar segments 10–17 was replaced by the anterior nerve cord from segments 4–11.

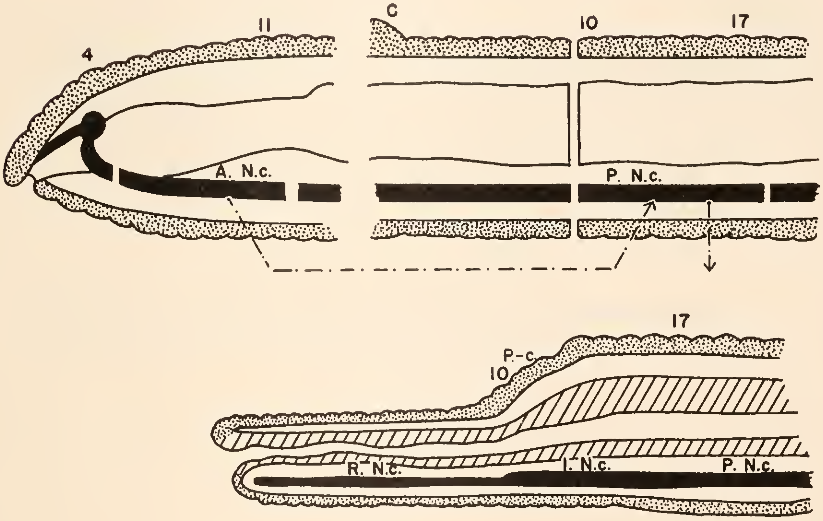

Fig. 4. Nerve cord of post-clitellar segments 10–17 is replaced by anterior nerve cord of segments 4–11. Result, heteromorphic tail. Implanted anterior cord has made connection with posterior cord.

The implant was made in normal orientation. After the healing of the posterior incision, simple transection was made just anterior to it, between post-clitellar segments 9 and 10. Examination at the end of two and one-half months reveals the presence of a heteromorphic tail of about twenty-five segments. New nervous material extends to the tip of the regenerate. Because of the bending of the worm, no one section shows both the anus and portions of the new and the implanted nerve cord.

Type 2. Nerve cord in head-forming region replaced by nerve cord from tail-forming region.

Case 61 (*Figs. 5 and 13*).—A median longitudinal incision was made on the ventral surface of the thirteenth and fourteenth anterior segments. This permitted severing the cord between these two segments. Anterior to the fifth anterior segment the cord was grasped firmly and pulled from segments 5–13. The nerve cord from post-clitellar segments 14–22 was pulled, in normal orientation, into these anterior segments by the use of silk thread and a long needle. The first four head segments, including the nervous organs, were discarded. At the end of two and one-half months the worm was fixed.

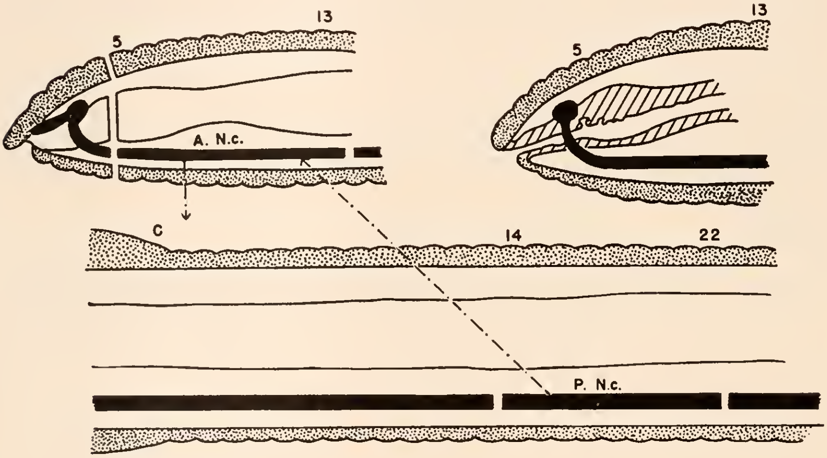

FIG. 5. Anterior nerve cord of segments 5–13 is replaced by nerve cord of post-clitellar segments 14–22. The first four segments are discarded. A new brain, commissures, three new segments, and a prostomium are regenerated.

A new head with brain, commissures, and oral opening is evident. Three segments and a prostomium are new. The brain lies in the third new segment.

In Case 103, the removal of the anterior nerve cord from segments

6–12 was following by the implantation, *in reversed orientation,* of the nerve cord from post-clitellar segments 13–27. In two and one-half months a brain, lacking commissures, formed independently of the implanted cord. An anterior perforation of the gut to the outside did not occur.

Series C. Body Wall

Type 1. Segments from head-forming region, including gut but lacking the central nervous organs, sewed in normal antero-posterior orientation to anterior surface of a post-clitellar segment; protruding nerve cord of host component pulled into graft.

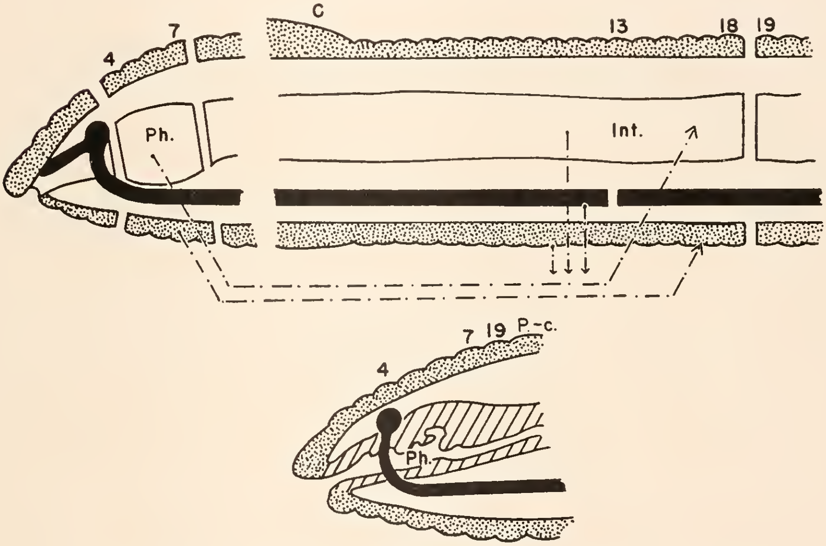

FIG. 6. Anterior segments 4–7, lacking the nervous parts, are grafted, in normal orientation, to anterior face of nineteenth segment behind the clitellum. The posterior nerve cord is drawn into the graft. Complete head regeneration results.

Case 47 (*Figs. 6 and 18*).—The anterior segments 4–7, including the digestive tube, but lacking the nerve cord, were sewed in normal orientation to the anterior face of the nineteenth post-clitellar segment. The nerve cord of post-clitellar segments 13–18 was pulled into the graft in the position occupied formerly by the anterior nerve cord. Two and one-half months later the worm was fixed.

Investigation reveals a regenerate possessing two segments, a prostomium, an oral aperture, a brain located in the third segment (first of the graft or fourth of the original worm), and commissures extending down to the nerve cord. The pharynx communicates with the intestine by means of a short portion of oesophagus.

In this case occurs the same combination of parts as in Series B, Type 2. In both, the posterior nerve cord participates in head regeneration.

Type 2. Anterior body-wall rings from head-forming region slipped over intestine back of clitellum (" sleeve operation ");

(*a*) No central nervous organs present within transplant (" sleeve ").

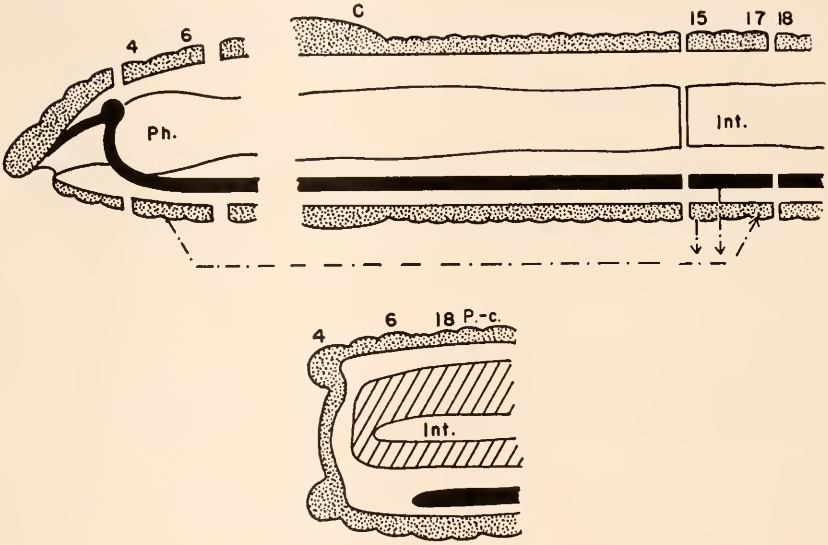

FIG. 7. A sleeve of anterior body-wall segments 4–6 is grafted over intestine of post-clitellar segments 15–17. The operated region contains no central nervous parts. Result negative.

Case 37 (*Figs. 7 and 15*).—A sleeve consisting of anterior body-wall segments 4–6 was slipped over the intestine of post-clitellar segments 15–17. All central nervous parts in the region of operation were discarded. The sleeve was normally oriented and sewed to the anterior face of the eighteenth post-clitellar segment. At the end of two months the worm was fixed. The sections show no activity except wound closure and healing.

In Case 17 the resulting conditions are somewhat different. The operation was the same as that above (Case 37) except that the sleeve was slipped over the intestine of post-clitellar segments 12–14 instead of 15–17. The length of time between operation and fixation was the same in both. In Case 17, however, a brain formed without any relation whatever to the ventral nerve cord. No commissures were evident; there is no anterior opening of the gut to the outside.

Cases 78 and 79 differ from both of the preceding in that the posterior nerve cord grew forward almost to the end of the sleeve.

Except these last three cases, all of this type agree in result with that of Case 37.

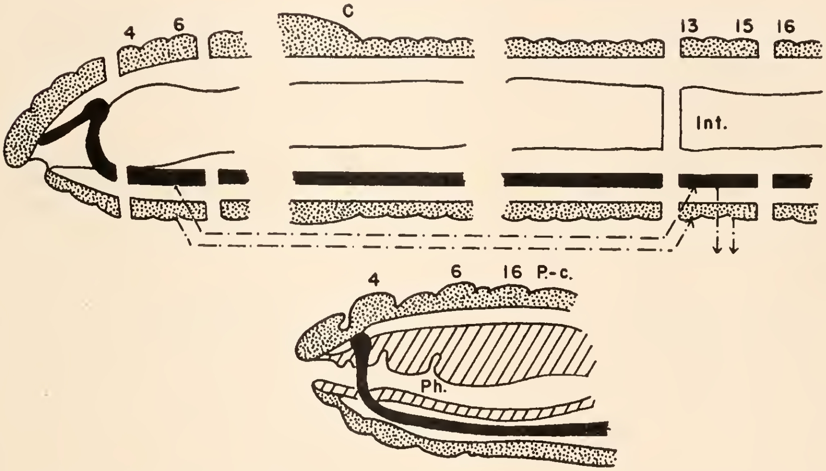

FIG. 8. A sleeve of anterior body-wall segments 4–6, including nerve cord, is grafted over intestine of post-clitellar segments 13–15. Head regeneration results.

(*b*) Anterior nerve cord present within graft.

Case 65 (*Figs. 8 and 14*).—After discarding the body wall and nerve cord of post-clitellar segments 13–15, a sleeve of anterior body-wall segments 4–6, together with their nerve cord, was slipped over the protruding intestine and attached (by stitches) in normal orientation to the anterior face of the sixteenth post-clitellar segment. At the end of two and one-half months the worm was fixed. Sections (Fig. 14) show a well-formed brain, commissures, typical pharynx epithelium, and an anterior opening of the gut to the outside.

Case 42 (Fig. 11) shows a regenerating brain and commissures, and an imperforate stomodeum. Figure 10, from another section of the same worm, shows the close relationship between the newly-formed brain and the epidermis.

(*c*) Posterior cord drawn forward into graft.

1. " Sleeve " placed in normal orientation.

Case 27 (*Figs. 9 and 19*).—A sleeve of anterior body-wall segments 4–6 was slipped, in normal orientation, over the intestine and nerve cord of post-clitellar segments 10–12. At the end of two months the worm was fixed. Sections show a new head with brain, commissures, a small amount of differentiated pharynx epithelium, and an oral opening.

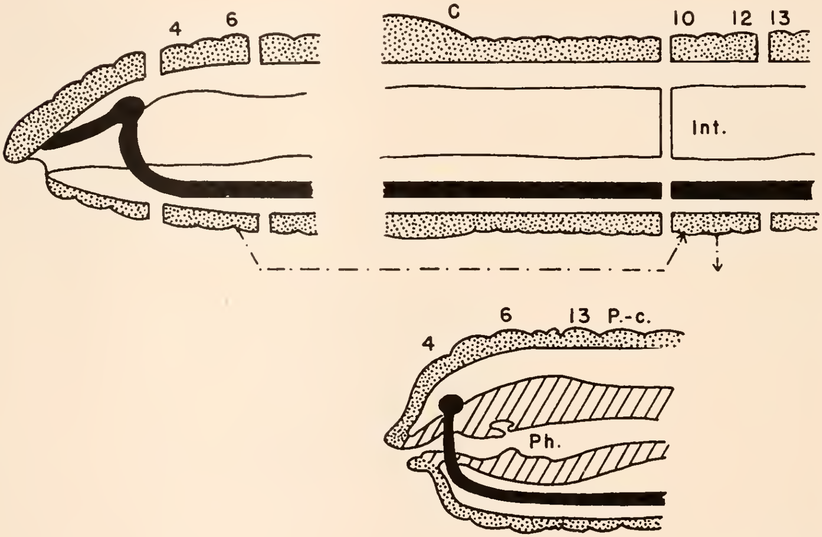

FIG. 9. A sleeve of anterior body-wall segments 4–6 is grafted over intestine and nerve cord of post-clitellar segments 10–12. Result, head regeneration.

With the exception of the oral opening, Case 32 possesses all the regenerated head parts noted in Case 27. The question of the origin of the pharynx epithelium will be discussed in another section of the paper.

Case 74 is another which clearly demonstrates the ability of the posterior nerve cord and intestine to participate in head formation when combined with anterior body wall.

The results of the three cases cited above, which agree essentially with those of seven additional cases, appear in Table I.

Three variations in the operative procedure, not included in Table I, showed different results. In Cases 75 and 83 the sleeve was oriented so that its dorsal side coincided with the ventral side of the host. As a result, the posterior nerve cord, pulled into the dorsal side of the sleeve at the time of operation, became attached dorsally by strands of connective tissue. No regeneration occurred.

Table I

Explanation of Symbols

	Anterior	Posterior
Body Wall	W	w
Gut	G^P (pharynx)	g^i (intestine)
Nerve Cord	N	n

	Combination	Number of Cases	Result
Series A			
Type 1	wG^P	13	No regeneration
Type 2	wG^PN	8	No regeneration
		1	Head
Type 3	wG^Pn	5	No regeneration
Series B			
Type 1	wg^iN	7	No regeneration
		1	Heteromorphic tail
Type 2	WG^Pn	6	Head
Series C			
Type 1	WG^Pn	5	Head
Type 2			
(a)	Wg^i	3	No regeneration
		1	Brain only
		2	Proliferation at end of nerve cord
(b)	Wg^iN	3	Head
(c)			
(1)	Wg^in normal orientation	10	Head
(2)	Wg^in W reversed antero-posteriorly	2	Results negative. Figure 17 shows slight regeneration.

In Case 44 the sleeve was oriented so that its dorsal side was lateral with reference to the dorsal side of the host component. At the end of a month and a half the posterior nerve cord had grown laterally to meet or give rise to new nerve material lying in the lateral plane (dorsal portion of sleeve).

2. " Sleeve " placed in reversed antero-posterior orientation.

Case 72 (*Fig. 17*).—A sleeve of anterior body-wall segments 4–6 was slipped over the intestine and nerve cord of post-clitellar segments 25–27. In contrast to previous cases, however, the sleeve was reversed so that segment 4 of the sleeve was sewed to the anterior face of post-clitellar segment 28. This left segment 6 of the graft in the anterior position. Normal dorso-ventral orientation of the graft was preserved. At the end of two and one-half months the worm was fixed.

Figure 17 shows a very slender anterior regenerate in the form of a finger-like projection. The nerve cord has grown forward and enters the base of the projection.

The results in this and in the one other case are the same; the activity of the nerve cord is confined entirely to the ventral side.

Out of more than four hundred worms used in the various experiments, only a small proportion survived. The significant results are summarized in Table I.

Discussion

The Rôle of the Digestive Tube in Regeneration

No experiment was performed with the express purpose of finding out whether or not regeneration would occur in the absence of the digestive tube at the wound region. The work of Morgan (1902) and Hunt (1919) shows that a head may begin to regenerate before the digestive tube, removed from the region just behind the plane of transection of the body, has grown forward to the wound region. In all the experiments performed in the present study some part of the digestive tube was present at the wound surface.

The pharynx alone, or in conjunction with the posterior nerve cord, failed to evoke head regeneration when implanted back of the clitellum. In like manner, the cases involving the combination of pharynx and anterior nerve cord in the posterior position are negative with the exception of Case 84. In this case, as already stated, there is a close relationship between the new nervous tissue and the pharynx epithelium (Figs. 20, 21). The characteristic columnar condition of the epithelium in this region has been lost. The layer, lacking a basement membrane, is made up of flattened cells with large, spherical nuclei. These epithelial cells merge uninterruptedly into the nerve cells so that there is no sharp demarcation between the epithelial layer and the brain anlage. Mitoses are abundant. There is very little evidence of proliferation from the old nerve cord. The relatively great distance of the epidermis from the brain (Fig. 20), and the absence of mitoses in the epidermis preclude any idea of contribution to brain formation from this source. The similarity of the pharynx epithelial and the prospective brain cells, their close proximity, and the abundance of mitotic figures indicate that, in this case at least, the pharynx epithelium has contributed to brain formation.

The apparent manner of origin of the brain in Case 84 is very similar to that described by Nuzum and Rand (1924). As early as a week following removal of the brain, without removal of any other part, they found groups of deeply-staining cells lying just above and in close relation to the pharynx epithelium. According to them, " These cells, containing large nuclei with prominent nucleoli, are similar to the cells of both pharyngeal and epidermal epithelia. Their position, corresponding precisely to that occupied by the obvious brain fundament of later stages, as well as their character, marks them as the early brain fundament " (p. 218). Siegmund (1928) suggested that the pharynx may be concerned in the formation of a brain which arises independently of the nerve cord.

It is possible, then, that the pharynx, ectodermal in ontogeny, may have some head-determining capacity. Results obtained in Series C, Type 2, show that the intestine can participate in head regeneration (Figs. 11, 14, 19), and that the epithelium of the intestine can dedifferentiate and redifferentiate into pharynx epithelium. Case 32 gives evidence that the new pharynx epithelium is derived from the intestine since there is no oral perforation and hence no possibility of contribution to pharynx formation by stomodeal epithelium. The endodermal origin of pharynx epithelium in regeneration is described by Hescheler (1898, p. 581) : " Das regenerierte Pharynxepithel geht aus den Zellen des alten Darmes hervor." Apparently, then, the pharynx is not essential to head regeneration even though it may, under some circumstances, be capable of giving rise to a brain.

The Rôle of the Nerve Cord in Regeneration

Morgan (1902, p. 571) maintained that the " cut-end of the nerve-cord is necessary at a cut-surface in order that a new head may develop." Bailey (1930) observed that regeneration does not occur when the nerve cord is looped back so that its cut end is remote from the wound. Results obtained by Goldfarb (1914), Siegmund (1928), Avel (1932), Crowell (1937) and Bailey (1939) show that the presence of the nerve at the wound surface is not essential to head regeneration; a brain may form independently of primary connection with the nerve cord. In the present work two instances of this sort, described above, were obtained. In Case 17, the sleeve of anterior body wall, grafted over the intestine back of the clitellum, contained neither anterior nor posterior nerve cord, yet a new brain formed in the anterior segment of the sleeve. Its close relation to the epidermis and the similarity between the cells of the brain anlage and the flattened epidermal cells give evidence that this brain was derived from the epidermis of the transplant.

Case 103 consisted of the transplantation of posterior nerve cord, in reversed orientation, to the head-forming region. As in Case 17 above, a brain formed showing close relationship to the epidermis but having no cellular connection with the implanted cord.

In all such cases where a brain develops independently of cellular connection with the nerve cord, there is the possibility that the cord exerts an influence through some neurohumor-like substance not observable in histological studies.

Especially significant are the sleeve cases and the cord transplantation experiments which demonstrate that the posterior nerve cord, usually associated only with regeneration of normal or of heteromorphic tails, can participate in head regeneration, and that the anterior nerve cord from the head-forming region can participate in the formation of a heteromorphic tail. Nervous structures of new heads in whose regeneration either anterior or posterior cord has participated show evidence of cellular proliferation in the old nerve cord. It is evident, however, that the nerve cord does not determine the form of the regenerate, and so can not be of the nature of an organizer.

The importance of the part played by the brain cannot be denied. The establishment of a brain anlage is always the first event in head regeneration. If, however, the nerve cord is concerned in this establishment, the posterior nerve cord is just as effective as the anterior nerve cord. We must look elsewhere for a specific *head* determiner. As suggested by Crowell (1937), it is not improbable that the new brain, however determined itself, may act as organizer during the further course of head regeneration.

The Rôle of the Anterior Body Wall in Regeneration

According to Hescheler (1898, p. 580) the epidermis is the chief source of cells giving rise to regenerated nervous material. He reports: " Während der ganzen Dauer der Bildung des neuen Centralnervensystems war keine grössere zusammenhängende Abspaltung von Epidermiszellen, sondern nur Zuwanderung einzelner Zellelemente zu beobachten." While Rand (1901) agrees with Hescheler that the epidermis is an important source of nervous material, Bailey (1930) observes no cells coming from the epidermis, and Schwartz (1932) claims that the connective tissue is the source of new cells in the cerebral ganglia. The observations made in the present study agree with those of Hescheler and Rand.

The rôle of the body wall is clearly seen in those cases where a sleeve of anterior body wall is grafted into the post-clitellar region in normal orientation with relation to the host component. A combination of such a sleeve, either anterior or posterior nerve cord, and intestine always evokes head regeneration. Attention has been called to Figure 17 (Case 72) which shows the negative results obtained when a sleeve of anterior body wall is grafted in reversed polarity over intestine and posterior nerve cord. This case, as well as the one other, involves the same combination of parts which, when all are in normal orientation, always produces a head.

Most of the normally oriented cases studied in this work represent such an advanced condition of regeneration that it is difficult to ascertain by what method the sleeve determines the form of the regenerate. Two cases, representing early stages in head regeneration, will be considered in more detail.

Case 17 was discussed briefly in connection with the rôle played by the nerve cord. The sleeve of anterior body-wall segments, enclosing the intestine back of the clitellum, contained neither anterior nor posterior nerve cord. The anterior end of the sleeve, at first open, closed in healing. The anterior cut end of the intestine likewise closed. Between the healed and closed anterior ends of body wall and intestine lies a cicatricial mass in which is embedded a brain anlage lying close to the epidermis. In the vicinity of the brain the epidermal layer, made up of flattened cells, is thinner than elsewhere. It is concave toward the brain, suggesting the beginning of a stomodeal invagination. The cells of the brain anlage, epidermis, and thin intervening mass are similar, all being of the epithelial type with large spherical nuclei and prominent nucleoli.

Case 42 (Figs. 10 and 11), in which an anterior body-wall sleeve and anterior nerve cord were grafted over the intestine, shows a developing brain, commissures, and an imperforate stomodeum. The relation between the brain and an inpocketing of the epidermis is seen in Fig. 10. Although ordinarily made up of columnar cells, the epidermal layer in this region consists of flattened cells and, furthermore, lacks the usual basement membrane. The cells appear to be undergoing a process of redifferentiation, a process marked by active mitosis and a distinct migration of the newly-formed cells to constitute the regenerating brain. This migration gives, in places, the effect of "streaming" of cell masses. The evidence afforded by this and the preceding case makes it clear that the epidermis of the anterior body wall initiates head regeneration by contributing cells which go to make up the brain. The fact that head regeneration has never been observed to take place in the absence of a brain anlage indicates that the brain, once established, evokes further and complete development of the head.

The question may now be asked, in what region of the anterior body wall is located the seat of brain determination, the dorsal portion, the ventral, or the lateral portion? With the exception of number 84, in which the brain anlage bears a close relation to the pharynx epithelium, all cases in which brain formation has been described indicate that the dorsal portion of the body-wall sleeve is the site of determination. In cases 75 and 83 the sleeve, ordinarily dorso-ventrally oriented with reference to the host, was oriented so that its dorsal side coincided with the ventral side of the host. This resulted in the apposition of the end of the posterior nerve cord and ventral side of the intestine against the dorsal side of the sleeve. Although giving no evidence in favor of the dorsal side as the region of determination, the fact that no regeneration occurred suggests that the ventral side of the sleeve, placed in the dorsal position, plays no rôle in the initiation of brain formation.

In Case 44 the sleeve was oriented so that its dorsal side was lateral with reference to the dorso-ventral axis of the host. Sections reveal that the posterior nerve cord has grown forward and laterally from its ventral course to meet or give rise to new nerve material lying in the lateral plane (dorsal part of sleeve). If the new material comes partly or even entirely from the old cord, then apparently the direction of its regeneration has been determined by the misplaced dorsal side of the graft. If the new material has its origin in the dorsal region of the graft, it and the old cord have advanced toward each other and united. According to Joest (1897), if an earthworm be transected and the two parts grafted together so that they are not properly oriented, the ends of the nerve cord, if not too far apart, will grow toward each other and re-establish connection.

In conclusion, it may be said that, in head regeneration, the brain, once established by the epidermis of the dorsal body wall, takes over the direction of further head development including the establishment of commissures and their connection with the nerve cord. We may thus regard the dorsal epidermis of the anterior body wall as the primary head determiner, and ascribe to the brain the rôle of secondary organizer.

SUMMARY

1. The presence of pharynx at a wound region is not essential to head regeneration even though it may, under some circumstances, be the source of nerve material (brain).

2. While obviously not a head determiner, the intestine can participate in head regeneration apparently as readily as the pharynx can. Intestinal epithelium can redifferentiate into characteristic pharynx epithelium.

3. The presence of nerve cord at a cut surface is not an essential factor in head regeneration since a brain may develop independently of any cellular connection with the cord. There may, however, be some hormonal relation between the nerve cord and regeneration.

4. The nerve cord does not determine the type of the regenerate for:

(*a*) The anterior (cephalic) nerve cord can participate in heteromorphic tail formation as well as in head formation.

(*b*) The post-clitellar cord (within the first twenty-five segments behind the clitellum) can participate in head formation as well as in normal and heteromorphic tail formation.

5. The "sleeve" operation, consisting in grafting over the intestine, in normal orientation, segments of anterior body wall containing either no central nervous parts, or anterior or posterior nerve cord, was invariably followed by head regeneration except in most of those cases in which no nerve cord was initially present. The "sleeve" cases afford strong evidence that the epidermis of the anterior body wall, being the source of the brain anlage, contains the primary determiner of head regeneration, and that the seat of this determination is probably located in the dorsal part of the epidermis.

ACKNOWLEDGMENT

It is a pleasure to express appreciation to Professor Herbert W. Rand, who suggested this problem and under whose helpful and critical supervision the work was performed.

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PLATE I

Explanation of Figures

Abbreviations used in plate figures:

Br.	brain
B.v.	blood vessel
I. N.c.	implanted anterior nerve cord
Int.	intestine
N.c.	nerve cord
Ph.	pharynx
R. N.c.	regenerated nerve cord

10. Case 42. High-power view of brain a few sections from that shown in Fig. 11 of the same case. Note proximity of brain to inpocketing epidermis. × 290.

11. Case 42. Regenerating head resulting from combination of a sleeve of anterior body-wall segments (4–6), anterior nerve cord, and intestine. Note imperforate stomodeum. × 29.

12. Case 77. Pharynx implanted thirteen segments behind the clitellum. No nerve cord within operated region. No regeneration. × 34.

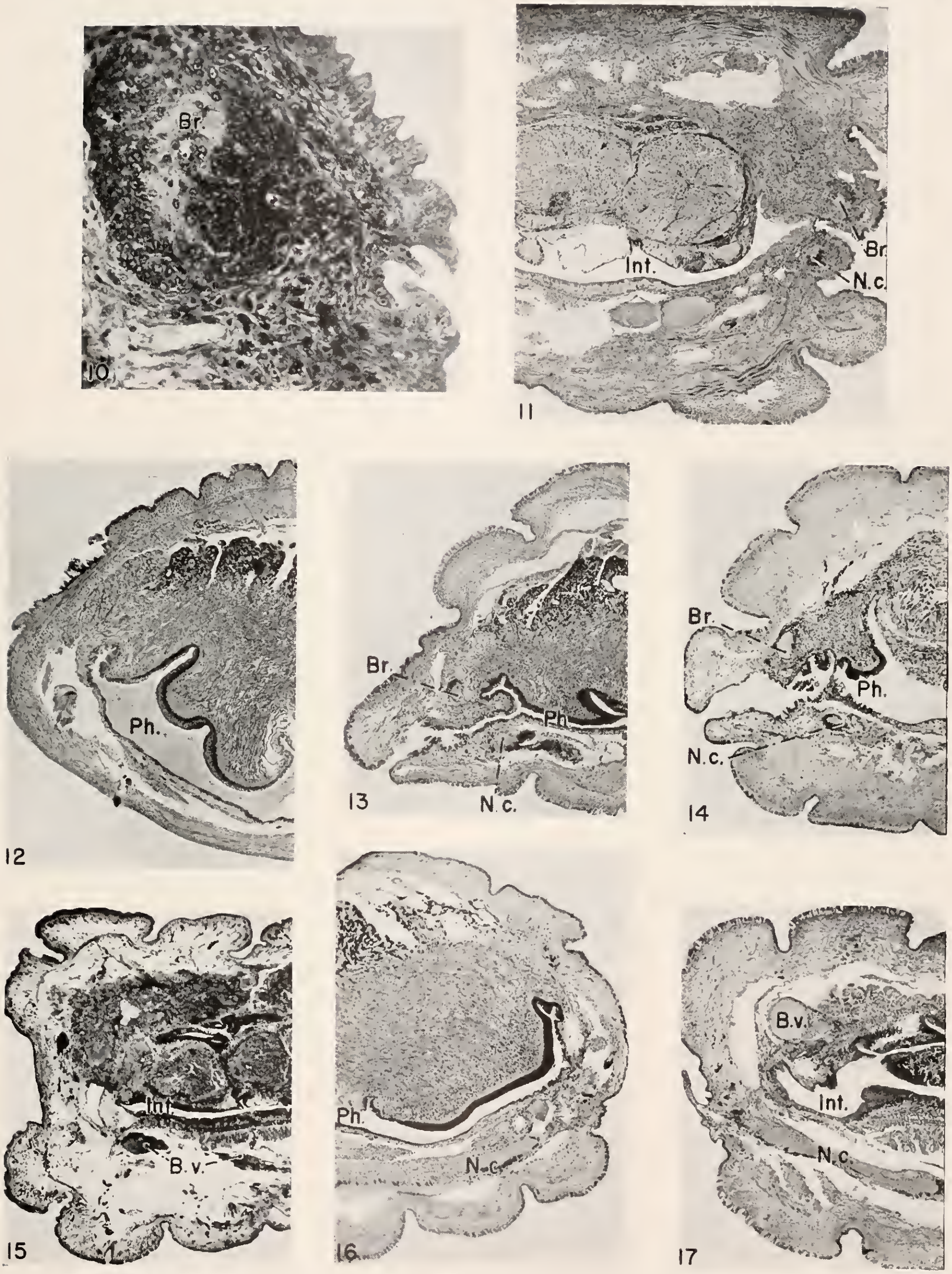
13. Case 61. Head regeneration occurred when posterior nerve cord replaced anterior cord. × 35.

14. Case 65. Regeneration of a head following graft of sleeve of anterior body-wall segments (4–6) and anterior nerve cord over intestine. Other sections show stomodeal perforation complete. ×31.

15. Case 37. Sleeve of anterior body-wall segments (4–6) grafted over intestine. No nervous parts included. Regeneration did not occur. × 24.

16. Case 68. Pharynx implanted back of clitellum. Posterior nerve cord left in position. No head regeneration. × 36.

17. Case 72. Sleeve of anterior body-wall segments (4–6) grafted in reversed antero-posterior orientation over intestine and nerve cord back of clitellum. No head regeneration. × 33.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Br.
11
Br.
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Ph.
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14
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15
Int.
B.v.
16
Ph.
N.c.
17
B.v.
Int.
N.c.

PLATE I

PLATE II

Explanation of Figures

18. Case 47. Regeneration of head following graft of anterior segments (4–7) to posterior portion of worm. Posterior nerve cord drawn into graft, replacing anterior cord. × 33.

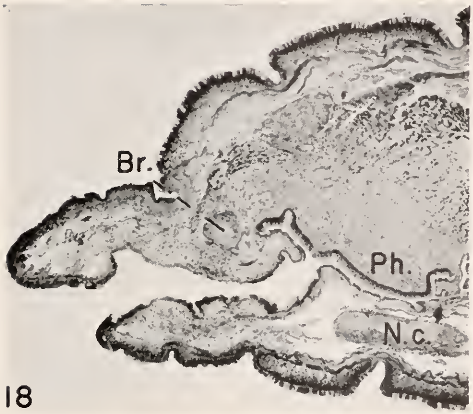
19. Case 27. Head regeneration following graft of sleeve of anterior body-wall segments (4–6) over intestine and nerve cord back of clitellum. × 45.

20. Case 84. Regeneration of a brain following implantation of pharynx and anterior nerve cord behind the clitellum. × 38.

21. Case 84. Enlargement of that portion of Fig. 20 inclosed within square. Note flattening of cells of pharynx epithelium and the resemblance of its cells to the nerve cells. × 304.

22. Case 11. Pharynx and anterior nerve cord are implanted back of clitellum. Some forward growth of nerve cord but no brain anlage. × 42.

23. Case 60. A heteromorphic tail formed when anterior nerve cord replaced posterior cord back of clitellum. × 33.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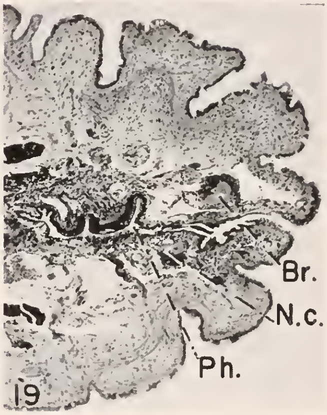
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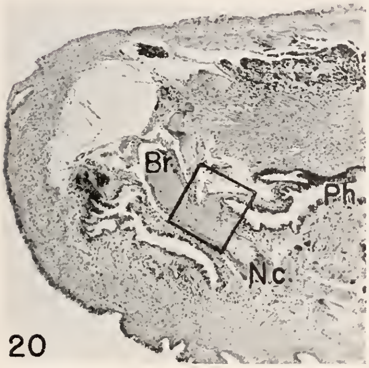
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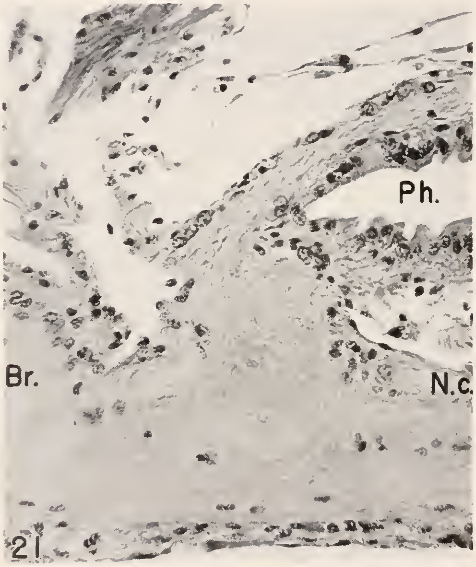
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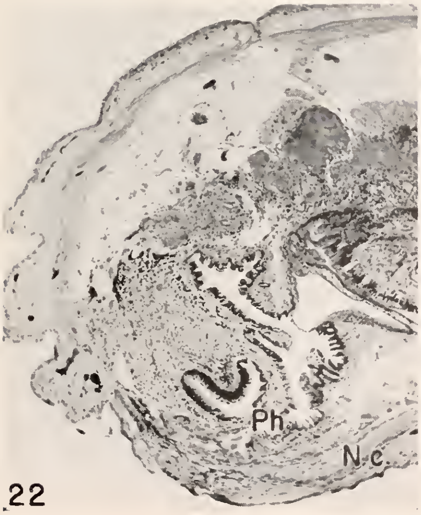
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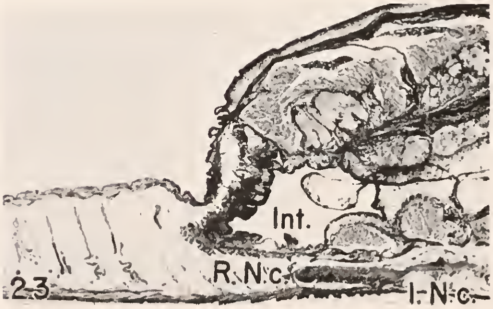
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PLATE II

BEHAVIOR OF THE CELL SURFACE DURING CLEAVAGE

III. On the Formation of New Surface in the Eggs of Strongylocentrotus Pulcherrimus[1]

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In the preceding paper of this series (1938), it was shown that the surface of a sea urchin egg cleaving in Ca-free medium is stretched during the process of segmentation to cover the two resulting blastomeres, but that during the interkinetic period, after cleavage has been completed, this previously existing surface is reduced in area, and an apparently new surface is formed in the region involved in the cleavage furrow. This conclusion was drawn from numerous observations that adhering kaolin particles can be found at any point on the surfaces of the two blastomeres at the time when cleavage is completed, but that following this, the particles slowly retreat from the cleavage furrow region, and before the end of interkinesis this area is invariably quite devoid of particles.

Since this conclusion depended solely upon observations of particles attached to the outside of the denuded egg, it was obviously desirable to examine the cortical protoplasm, in order to rule out entirely the possibility that the particles were attached to some investing membrane whose behavior might be different from that of the true protoplasmic surface. An opportunity to settle this point was offered by the method of Motomura (1935), who reported observations on the behavior of pigment granules in the cortex of *Strongylocentrotus pulcherrimus* egg, which, he discovered, could be clearly seen with the aid of a blue filter. His conclusions, however, were considerably at variance with those of the authors, and it therefore became more interesting than ever to combine studies of the the particle-bearing surface layer with those of the granule-containing cortex. The results of this work are presented herewith in three sections. In the first will be presented a description of the pigment granules of *Strongylocentrotus pulcherrimus* eggs as well as some correlated observations on *Arbacia* eggs; the second part will deal with observations on the behavior of the *Strongylocentrotus* granules during cleavage and interkinesis; and the third, with combined observations of particle migration and granule behavior.

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[1] This study was assisted in part by a grant from the Foundation for the Promotion of Scientific Research of Japan.

Pigment Granules of Strongylocentrotus, Arbacia pustulosa, and A. punctulata

For the original account of the pigment granules of *Strongylocentrotus pulcherrimus* eggs, Motomura's 1935 paper may be consulted. Briefly, they are small, orange in color, and poorly differentiated from the surrounding protoplasm when viewed with white or yellow light. If, however, a Wratten No. 49 filter is interposed between the eggs and the light source, the granules become more sharply differentiated, appearing dark-red or almost black with the light source used by the authors [2] (*see* Motomura, 1935, Fig. 64). In sections of eggs fixed by chrome-osmium mixtures or by chrome-formol fixatives with later osmication, the granules are strongly blackened.

The position and consequent visibility of these granules vary with the physiological condition and stage of cleavage of the eggs. For this reason, the results of three methods of observation—i.e., of living eggs with white and with filtered light, and of stained sections—have been combined to form the final picture of the distribution of the granules in unfertilized, fertilized and cleaved eggs.

Unfertilized Eggs in White Light.—Examination of the cortical region of living, unfertilized eggs under high magnification with artificial light or with sunlight (Plate I, *A*–1) shows structurally undifferentiated protoplasm of a light orange color extending to the "protoplasmic surface film" (*see* Chambers, 1938).[3] If the focus is raised, the surface of the egg appears to give off an orange color, but this color is diffuse and cannot be definitely attributed to any particular granules.

Unfertilized Eggs in Filtered Light.—If a Wratten No. 49 filter is introduced into the optical system, the egg acquires a just-perceptible, dark-reddish line at the circumference of the largest optical section, and when the focus is raised to bring the upper surface into view, scattered granules of this color appear, as nearly as can be determined, immediately below the protoplasmic surface film (Plate I, *B*–1).

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[2] Osram point-light bulb mounted in a Leitz-Wetzler lamp with a transformer of 6 volts, 5 amperes.

[3] Before Chambers' paper came to their notice, the authors had performed various simple experiments to determine the real existence of this extremely thin, transparent layer which was so consistently found to be present that it was for some time thought to be a refraction artifact. It is visible with direct and dark-field illumination on eggs in sea water and Ca-free medium, and on exovates formed by compressing either fertilized or unfertilized eggs in sea water. On eggs normally fertilized in sea water, the protoplasmic surface film can be distinguished lying beneath the hyaline plasma layer. If such eggs are transferred to Ca-free medium, the hyaline plasma layer dissolves away inside the fertilization membrane, leaving the underlying surface film as the only visible structure closely investing the egg. In only one case was it found possible to effect a change in the appearance of this film. When unfertilized eggs were compressed, and then released before they burst, the protoplasmic surface film on the contracted surface lost its usual perfect contour, appearing roughened and irregular.

Unfertilized Eggs in Section.—Unfertilized eggs treated with OsO_4 show a layer of blackened granules, which are distinguished from other granular inclusions by their slightly larger size, and lie so closely beneath the protoplasmic surface (Plate I, *C*–1) that they appear to be in contact with it.

Fertilized Eggs in White Light.—In less than two minutes after fertilization or after artificial activation, the egg in optical section presents a quite different picture, aside from the appearance of the fertilization membrane and the gradually thickening hyaline plasma layer. In white light, beneath the protoplasmic surface film, a zone of extremely finely granular (but not hyaline) protoplasm has appeared, apparently as a result of the centripetal migration, by approximately 1.5 μ, of the coarse granules which had been occupying this region (Fig. 1 and Plate I, *A*–2, *A*–3). This zone, which will hereafter be called the "extra-granular zone," gradually widens, reaching a width of about 2 μ before cleavage begins. Centripetal to the extra-granular zone and forming an outer boundary to the central heterogeneous granular protoplasm is a slightly irregular ring of larger, definitely orange granules, whose color is transmitted to the adjacent extra-granular zone. If the focus is raised, it can be seen that the upper surface no longer gives off the orange radiation which appeared in the unfertilized egg.

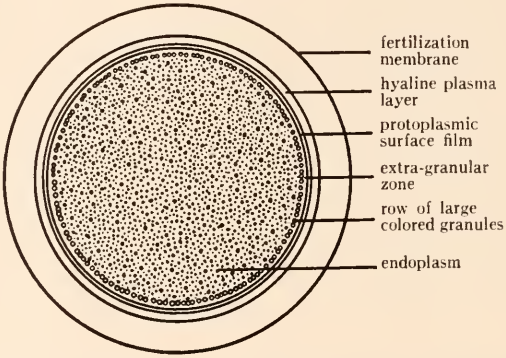

FIG. 1. Diagram of fertilized egg of *Strongylocentrotus pulcherrimus* in sea water.

Fertilized Eggs in Filtered Light.—When observed with filtered light, the fertilized egg in optical section is seen to have acquired a prominent ring of dark-reddish granules whose position corresponds to that of the orange granules seen with white light (Plate I, *B*–2). At the surface level, these present the same picture as those in the unfertilized egg, i.e., they lie scattered at random.

Fertilized Eggs in Section.—Osmicated fertilized egg sections show a row of strongly blackened granules at a corresponding distance within the egg cortex [4] (Plate I, *C*–2). On the basis of these observations and others presented below the authors are convinced that the granules which appear as the prominent ring in the fertilized egg move *inward* at fertilization from their previous position immediately inside the protoplasmic film, rather than *outward* from the endoplasm. The fact that their presence in the egg before fertilization can be detected only as a diffuse orange radiation can be explained on the ground that they are lying so closely in contact with the concave surface of the outer protoplasmic surface film as to be individually indistinguishable in optical section.

The conspicuous red granules of the *Arbacia* egg have been objects of interest for many years (Harvey, 1910; McClendon, 1910, et al.). It has generally been accepted that these granules move from the interior of the egg to the cortex at the time of fertilization, but this observation is difficult to prove. However, it is certain that a demonstrable rearrangement takes place during the four and a half minutes following fertilization in that the granules, which in the unfertilized egg appear to be located at various depths from the surface, move into a single layer in the now firmly gelated cortex (Plate II, *B*, *C*). This behavior is observed in the eggs of both *Arbacia punctulata* of Woods Hole and *A. pustulosa* of Naples. Although the details of the *Arbacia* granule movement are decidedly at variance with those which have been observed in *Strongylocentrotus*, the possibility seems to be worth considering that the rearrangement may be caused by inward migration, as is the case in *Strongylocentrotus*, rather than by the outward movement generally regarded as the sole process involved. This same change in distribution occurs after artificial activation.

Pigment which can be extracted from the tests of sea urchins is generally known as "echinochrome" after MacMunn's nomenclature, and it has been proved that the pigment is an oxidation-reduction dye (MacMunn, 1885, 1889; Griffiths, 1892; Cannan, 1927). Pigment which is contained in the eggs of several kinds of sea urchins including *Arbacia* ("arbacine" of Vlès and Vellinger, 1928) has also been identified as echinochrome (McClendon, 1912; Ball, 1934), and the observation that the granules move to the cell periphery after fertilization concurrently with a great increase in respiration lent further support to the conclusion that they perform a respiratory function (Runnström, 1928). Crude trials were made to determine whether observable changes take place either in the orange coloration or in the spectroscopic absorption

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[4] Because of the shrinkage of the eggs incident to fixing and sectioning as well as the difficulty of determining the exact level of section in a given case, and since the distances in question are very small, the making of precise measurement was rejected as involving too large an error to have any meaning.

PLATE I

A. Strongylocentrotus eggs in white light.
  A–1. Unfertilized egg. Notice that there is no differentiated structure inside the egg.
  A–2. Fertilized egg in Ca-free artificial sea water. Fertilization membrane has been removed mechanically and hyaline plasma layer has dissolved away. Note extra-granular zone around periphery and row of granules inside it. Dark appearance of granules is due rather to high refractivity than to intense color. Protoplasmic surface film is not clearly defined in this photograph.
  A–3. Fertilized egg in normal sea water, showing fertilization membrane, hyaline plasma layer, protoplasmic surface film, extra-granular zone and row of granules (cf. diagram, Fig. 1).

B. Strongylocentrotus eggs in filtered light—mixed culture of fertilized and unfertilized eggs in Ca-free medium. (Since sea-urchin eggs cannot be fertilized in Ca-free medium, there was no danger of contamination of unfertilized eggs by excess sperm from fertilized eggs. As extra precaution, eggs were well washed after fertilization and allowed to stand some time before being mixed with unfertilized eggs.) In order to insure easy differentiation between fertilized and unfertilized eggs, fertilization membranes were not removed, but were left in place in collapsed condition which results upon their introduction into Ca-free medium. This collapse of fertilization membrane permitted simultaneous focusing upon fertilized and unfertilized eggs, which is impossible when membranes are normally expanded. Aside from collapse of membrane and disappearance of hyaline plasma layer, appearance of eggs in sea water and Ca-free medium is identical.
  B–1. Unfertilized egg, showing absence of differentiation in granular cytoplasm.
  B–2. Fertilized egg, showing dark-red ring. (Outermost structure is collapsed fertilization membrane, beneath which protoplasmic surface film is poorly visible at right side. Hyaline plasma layer has dissolved away in Ca-free medium.)

C. Strongylocentrotus eggs in sections.
  C–1. Unfertilized egg showing row of osmium-blackened granules closely applied to cell surface.
  C–2. Fertilized egg with extra-granular zone and row of granules beneath it.

D. Strongylocentrotus egg in late interkinesis—Ca-free preparation showing difference between "old" surface on polar side of blastomeres provided with extra-granular zone, and new surface along cleavage furrow without extra-granular zone. Note high refractivity of newly-formed surface.

E. Strongylocentrotus egg in four-cell stage (Ca-free medium). Note that "old" surface appears only on outer sides of blastomeres.

F. Strongylocentrotus egg in section. Separated blastomere in which polar and furrow regions are clearly differentiated by contour, showing absence of extra-granular zone and presence of highly refractive membrane in furrow region.

G. Section of spherical separated blastomere of *Strongylocentrotus* in which direction of preceding cleavage is indicated by differentiated structures as in *F*.

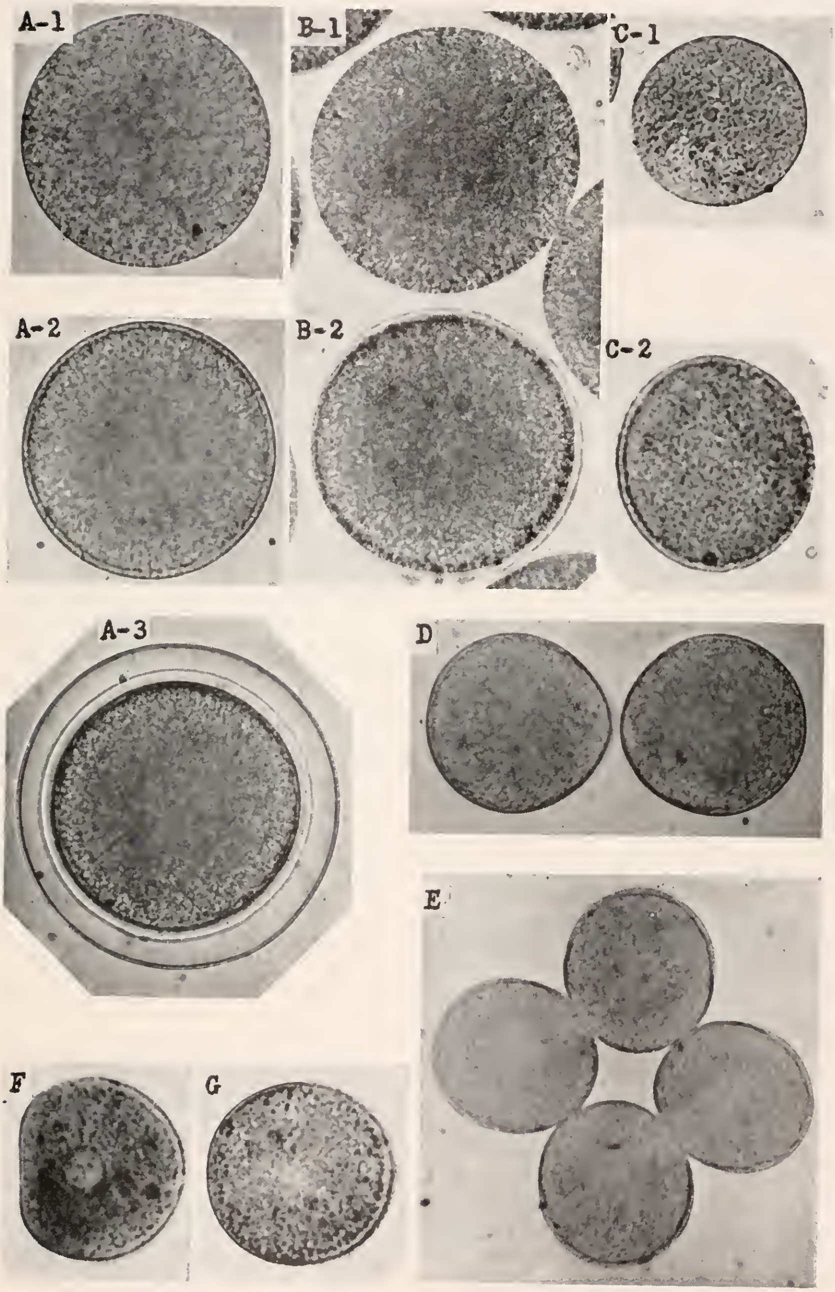
A-1
B-1
C-1
A-2
B-2
C-2
A-3
D
E
F
G

PLATE I

of the *Strongylocentrotus* granules under the influence of oxidants or reductants. Although it is still premature to make a final conclusion, at least in water extract and acetone extract of the pigment and in the pigment *in vivo*, no change could so far be detected, contrary to the case of the pigment extracted from the tests of this species (Suto, 1938).

A further marked difference in physiological function between these granules and those of *Arbacia* is found in the fact that these granules do not break down upon cytolysis or liberation into sea water, while the *Arbacia* granules do (Heilbrunn, 1928). However, the somewhat similar position and the fact that there is a remarkable change in distribution following fertilization suggest that there may be a closer relationship between them than is apparent from the existing data.

Behavior of the Cortical Elements in Cleavage

In this section, because of its importance in the later discussion, the behavior of the cortical elements in eggs cleaving in Ca-free artificial sea water will be described in detail.

The eggs of *Strongylocentrotus pulcherrimus* were prepared for observation by the following procedure: insemination was performed in a minimum of sea water, and when the fertilization membranes were par-

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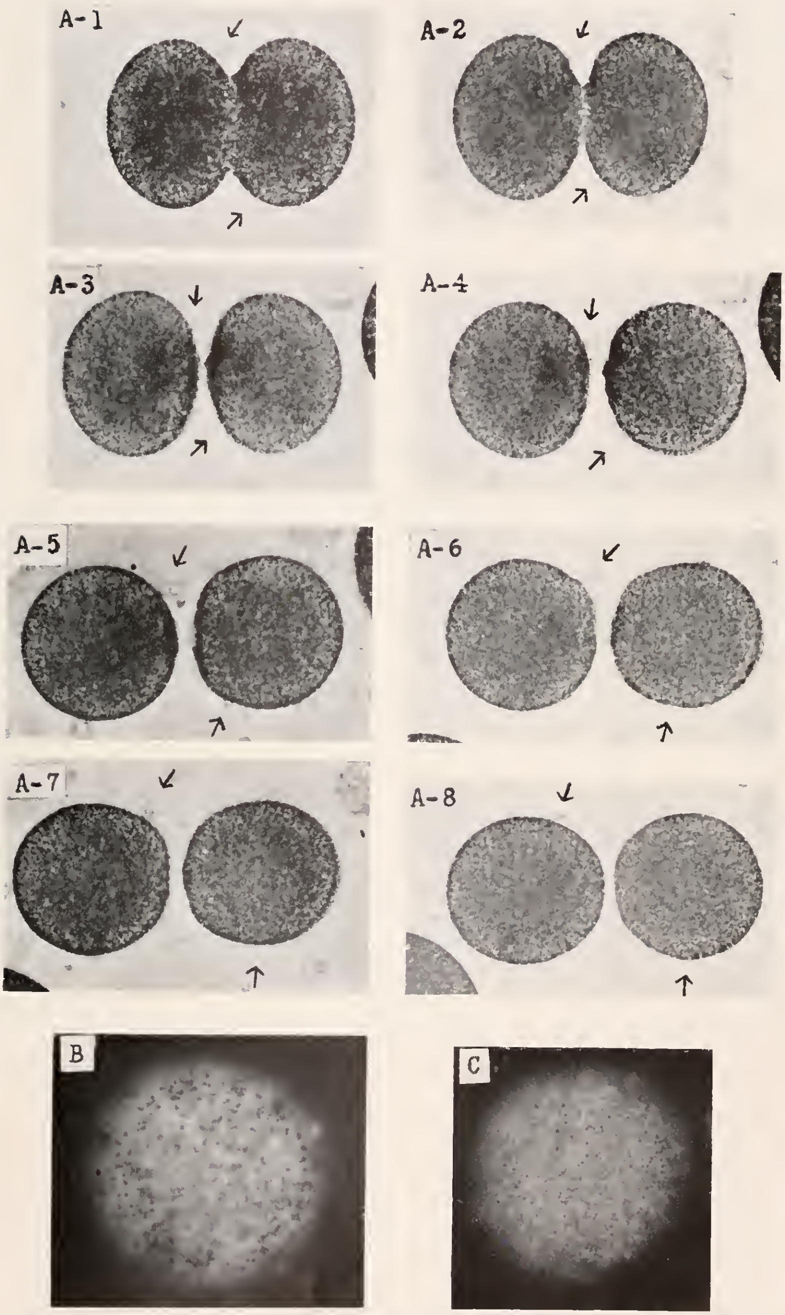
PLATE II

A. Consecutive photographs of division of *Strongylocentrotus* egg taken with direct sunlight through filter at low magnification, later enlarged. This series aims especially to show interrelation of dark-red ring and kaolin particles. (Room temperature 13° C., process plates used for photographing.)
  - *A–1.* Two kaolin particles stuck on sides of furrow (indicated by arrows). Note that dark-red ring completely covers surface.
  - *A–2.* One minute later. Furrow has deepened and particles are carried further into it.
  - *A–3.* Two minutes, thirty seconds later (than first photograph). Cleavage is complete. Note that dark granules are accumulating around point of last connection between blastomeres.
  - *A–4.* Three minutes, thirty seconds later. Blastomeres have rounded up.
  - *A–5.* Five minutes later. Dispersion of granules and migration of kaolin particles out of furrow have started simultaneously.
  - *A–6.* Six minutes later. First sign of opening of granular ring appears.
  - *A–7.* Seven minutes later.
  - *A–8.* Ten minutes later. New surface has fully developed. Note that kaolin particles are well outside furrow and dark granules are distributed only on polar sides of blastomeres.

B. *Arbacia pustulosa*, unfertilized egg in dark-field illumination. Surface view of egg, showing that pigment granules are at different depths from surface, so that simultaneous clear focus is not obtainable. (Note that this is reverse of case with *Strongylocentrotus*, in which granules are in single layer closely applied to protoplasmic surface film.)

C. Surface view of fertilized egg of *A. pustulosa;* pigment granules are now in a single layer.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A-5
A-6
A-7
A-8
B
C

PLATE II

tially raised (about 1 minute later), a mixture of equal parts of Ca-free artificial sea water and 0.53M (isotonic) KCl was added to the eggs. In this solution, the membranes became abnormally expanded and fragile, and were removed without injury to most of the eggs by the use of a fine pipette of the proper bore. Since too long a stay in the presence of an excess of KCl produces abnormalities in the eggs at the time of cleavage, they were returned to Ca-free sea water, washed repeatedly, and allowed to develop until just before the first cleavage. At this time, an equal amount of isotonic KCl was again added to the suspension (since it was found that, in this species, in Ca-free sea water alone the blastomeres tend to come into close contact during interkinesis, rendering observation difficult, while this does not occur if excess KCl is present, although in all other respects the cleavage picture is the same.)

In White Light.—As cleavage begins in *Strongylocentrotus* eggs, the mutual relations of the granular ring, the extra-granular zone and the protoplasmic surface film are maintained practically unchanged. However, as the furrow progressively deepens, the extra-granular zone at the polar regions[5] becomes noticeably narrower, presumably because of stretching (Fig. 2, *A*, *B*, *C*). About the time when the separation of the blastomeres is complete, the granules appear to accumulate to some extent in the cortical protoplasm forming the walls of the furrow, and, moreover, are not so uniformly equidistant from the surface in this region (Fig. 2, *D*). During the first part of the interkinetic period, the accumulation of granules in the walls of the furrows is gradually dispersed, and the extra-granular zone in the furrow region, which has become continuous by the complete severance of the blastomeres, is gradually opened, starting from this central point (Fig. 2, *D*, *E*). As it opens, its place is occupied by an increasing extent of surface which is remarkably similar to that of the unfertilized egg; i.e., no extra-granular zone is present, the orange granules are not separately distinguishable and the coarse granular endoplasm extends all the way to the surface film (Fig. 2, *E, F*). However, this outer membrane is sharply defined against the surrounding medium because of a high refractive index which is found only in this newly-formed surface, and is not characteristic of the polar surfaces of the blastomeres (Plate I, *D*) or of the unfertilized egg surface (Plate I, *A*–1).

In the second cleavage, a practically similar process is repeated, the formation of new surface taking place in such a way that the extra-granular zone comes to lie only on the outer surfaces of the four blastomeres (Plate I, *E*); and so far as the authors can determine, this is regularly kept up until the blastula stage (see Motomura, 1935, Fig. 71).

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[5] In the papers of this series, the authors have adopted the terminology of Chambers with respect to the topography of cleaving eggs; i.e., the furrow is referred to as the equator in defining the poles. (See diagrams in previous papers.)

In Filtered Light.—In the cleaving egg seen with filtered light, the dark-red ring covers the entire surface (Plate II, *A*–1, *A*–2). When

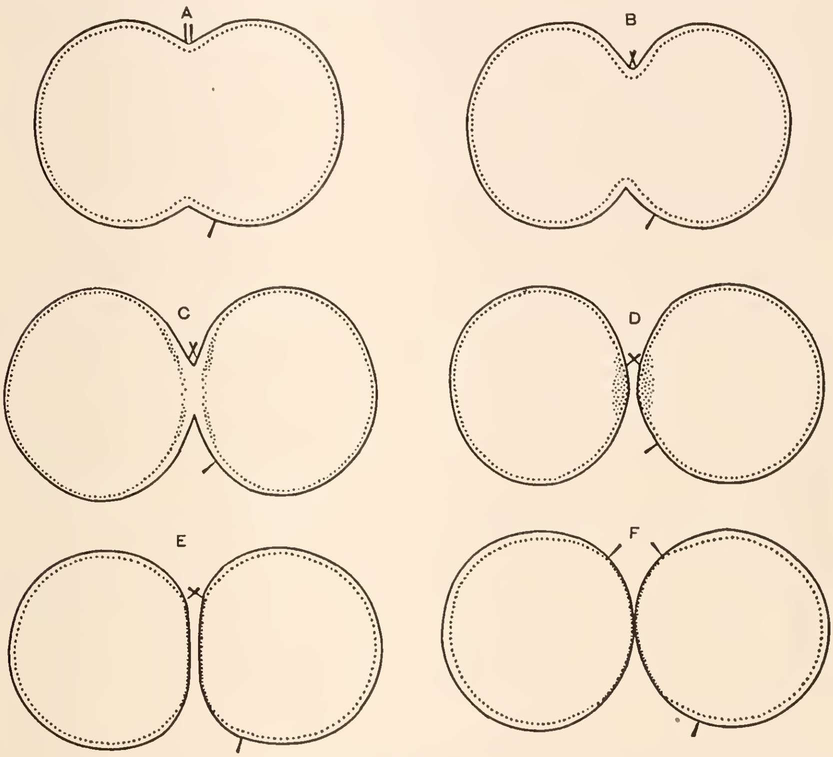

FIG. 2. Diagram of typical *Strongylocentrotus* experiment to show, simultaneously, behavior of kaolin particles adhering to surface, extra-granular zone and granular layer. (Changing outline of cleaving egg and positions of adhering particles are traced from photographs. Positions of granules are indicated by dots.)

A. Cleaving egg; kaolin particles are situated in furrow region; extra-granular zone has narrowed at poles, presumably because of stretching.

B. Kaolin particles are carried inward by deepening of furrow.

C. Extra-granular zone in furrow region widens, at poles becomes extremely narrow.

D. Extra-granular zone regains uniform width around blastomeres; granules are accumulated at point of last connection between blastomeres.

E. Formation in furrow region of new surface, lacking in extra-granular zone. Kaolin particles have been pushed out of furrow; accumulated granules have dispersed—they are now lined up in close proximity to newly-formed surface.

F. Late interkinetic stage; extra-granular zone over polar two-thirds of blastomeres has widened as though released from tension; upper particles are now well outside of furrow, at boundary between old and new surfaces.

the severance of the blastomeres is nearly completed, the dark-red ring in optical section becomes very thick at the sides of the furrow (Plate II, *A*–3, *A*–4); after cleavage is completed, it first regains a more uniform width all around the blastomeres (Plate II, *A*–5) and then breaks at the hyaline connecting stalk (Plate II, *A*–6, *A*–7) and becomes less and less a complete ring, finally including only about 65 per cent of the circumference of the blastomeres (Plate II, *A*–8). The surface where the ring has opened is characterized by the absence of the red granular ring, as is the case with unfertilized eggs. In the four-cell stage, the dark-red granules are distributed on the outer sides of the four blastomeres, corresponding exactly to the distribution of the extra-granular zone as seen in white light.

Sections.—In sections of eggs fixed with osmium mixtures, however, contrary to the expectation from observations in filtered light, there is a row of blackened granules extending completely around both the newly separated blastomeres and blastomeres in the interkinetic period. In the early stages of cleavage, at the poles, the ring is closer to the surface —i.e., the extra-granular zone is narrower than in the other regions; while in the cortex adjoining the furrow, the granules are less perfectly aligned, sometimes lying 5 or 6 μ inside the surface layer. In the later interkinetic stages, the ring of granules extends around the polar part of the blastomere section at a uniform distance from the surface as in the egg before cleavage. Around the periphery on the furrow side, this separating zone of protoplasm (extra-granular zone) is quite lacking, and the row of blackened granules is found closely applied to the surface membrane, as in unfertilized eggs. However, in this case, the granules appear to be more numerous, forming a continuous, highly refractive line (Plate I, *F, G*). This greater accumulation of granules in fertilized eggs than in unfertilized eggs may explain why this part of the blastomere surface is more clearly defined, in the living condition, than that of the unfertilized eggs.

When fertilized eggs are allowed to cleave in sea water, so that the fertilization membrane and hyaline plasma layer are intact and the blastomeres closely in contact as soon as the cleavage furrow is complete, it is impossible to distinguish either orange granules or dark-red ring along the contact surface (Motomura, Fig. 70). However, studies of sections reveal that, as is the case when cleavage takes place in Ca-free sea water, the extra-granular zone covers the entire surface before the complete severance of the two blastomeres, but that later it retreats from the furrow region while the row of granules is always encircling the endoplasm. From this, it must be concluded that the apparent absence of orange color in white light and of the dark-red ring in filtered light is controlled not by the actual absence of the granules but rather by the optical conditions imposed by the presence of the extra-granular zone or by the spatial relations of the blastomeres.

Another indication that the apparent absence of the dark-red granules in the walls of the cleavage furrow in sea water is an optical illusion arising from the very close association of the two surfaces is furnished by the observation that when two fertilized eggs with perfectly complete rings of dark-red granules are brought into contact, the granules appear to be absent from the parts of the egg peripheries which are in contact. Therefore, since the visibility of the dark-red ring with a filter appears to be largely dependent upon the optical conditions, it must be concluded that one cannot safely determine the presence or absence of granules on the sole basis of observations with the filter.

Summarizing this section, it can be said that direct observation of the cortex reveals, as was predicted in the previous paper from the behavior of the kaolin particles, that the existing surface of the uncleaved egg is pulled into the furrow during the process of segmentation, and that only when the interkinetic period is reached, does a new surface begin to be formed along the furrow sides.

Simultaneous Observation of Cortical Elements and Kaolin Particles Affixed to the Surface of Strongylocentrotus Eggs

Kaolin particles were added to a Ca-free culture of the eggs, prepared in the way described above. After thorough mixing, excess particles were removed by washing. Eggs were mounted in a deep hollow slide, a cover glass was fastened in place by means of vaseline, and the whole preparation was immersed in a larger volume of Ca-free medium to reduce local heating by the light source.

When photographs were taken with filtered light, in order to make the exposure time as short as possible, direct sunlight and a low magnification were used. An egg with suitably located particles was selected and photographed at intervals from before the completion of cleavage until nearly the end of the interkinetic period. Figures shown in Plate II are enlarged pictures of such a series. In these photographs, a clear image of the extra-granular zone was sacrificed in order to show the dark-red ring.

In these series, eggs were chosen with particles at the head of the cleavage furrow, because this is the spot where it is anticipated that the new surface will begin to appear. If so, the particles which are at the head of the furrow are expected to come to lie at the border between the old and new surfaces when the latter is formed. In filtered light, this border will be the place where, in optical section, the dark-red ends. In white light, such particles will be found where the extra-granular zone ends. This expectation has been borne out perfectly by many observations.

The unfailing coincidence between the position of such kaolin particles and the border line between the old and new surfaces (*see* diagram, Fig. 2, *E, F*) leads to the conclusion that the behavior of the kaolin particles can be taken as a direct indication of the behavior of the cortical material. Evidently, the kaolin particles must be adhering to the protoplasmic surface film, which is the outermost structure persisting in a Ca-free medium, and the above findings show that the protoplasmic surface film does not slip over the underlying cortical layer as does the hyaline plasma layer [6] (Dan, Yanagita and Sugiyama, 1937).

Discussion

In the preceding sections the observations have been presented in the light of the authors' interpretations. However, since Motomura's interpretations sometimes differ from those of the authors, a few words will be devoted to comparing the two.

In connection with cleaved eggs in sea water, Motomura concluded that the granules are present only along the free surfaces of the blastomeres and that they are absent along the contact surfaces,—a conclusion based on the fact that the dark-red granules in filtered light can be seen in the former position but not in the latter. So far as the observation is concerned, our finding coincides perfectly with Motomura's. However, if the identity of the dark-red granules and the osmium-stained peripheral granules in sections is assumed, as the authors are compelled to do from the coincidence in size, position and behavior between them, it must be concluded that the granules are present all over the surface at any time, and that it is the extra-granular zone which is present only on the free surfaces and not along the newly-formed surfaces (Motomura, Fig. 70). The authors' explanation of the observation is that the granules will take a dark-red color in filtered light anywhere and at any time, but when they are closely in contact with a surface, it is not possible to distinguish them. However, both sets of conclusions agree in saying that the part of the surface of cleaved eggs in which the dark-red granules are invisible in filtered light is a newly-formed surface.

Concerning the case in which eggs are kept in a Ca-free medium and are allowed to cleave in it, Motomura came to a rather confusing conclusion. Quoting him: " In short, the new cell boundaries are not formed in the calcium-free sea water in the course of the early cleavages. The surface of each blastomere is covered by the extension of the pigmented cortical cytoplasm. But, when the blastomere is replaced in the normal sea water, it regains the capacity of forming the new cell boundaries. In this case the blastomere is able to form the pluteus. When the egg is cultured successively in calcium-free sea water, no formation of the embryo in it is possible. In this case the distribution of the pigment granules is irregular " (p. 239). From this description, he apparently missed the formation of new surface in Ca-free sea water entirely. In the authors' experience, in order to observe the formation of new surface, special precautions are necessary to secure a good condition of the cultures. Overheating of the preparation by the light source or a slight shortage in the quantity of the culture medium at the time of observation immediately impairs the eggs. During the first cleavage, when such injury occurs, the eggs invariably stop their development at the stage in which the separation of the blastomeres is just completed and the granules are accumulated at the sides of the furrow (Plate II, *A*–3, *A*–4). Therefore, even as early as the beginning of the interkinetic period, it is possible to predict whether or not an egg under observation will undergo further cleavages. Cultures were encountered, especially toward the end of the season, in which all the eggs remained in this state. In such eggs, the granules sooner or later begin to clump together irregularly, and the eggs finally die. But so far as the authors are aware, as long as the condition of the egg remains favorable, each cleavage is regularly followed by the formation of new surface. Hence, it is impossible to accept Motomura's argument in the same chapter, that the failure of larvae in Ca-free sea water to develop into plutei is due more to the lack of new surface formation than to the falling apart of the blastomeres.

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[6] It incidentally follows that a new protoplasmic film must be produced over the newly formed surface of the blastomeres. This is to be expected from the previously reported observations, in which no intact protoplasmic surface could be found without the investing film.

The assumption that the granules under discussion are located only in the cortical layer and not in the endoplasm has already been made by Motomura on the ground that exovates do not contain the granules. This conclusion is tenable only under the circumstance that the granules do not break down when the cells are injured, unlike the case with *Arbacia* granules. The authors' further experiments with this view in mind, together with their study of sectioned eggs, support Motomura's contention.

The authors are happy to acknowledge their indebtedness to the Marine Biological Laboratory of Woods Hole and to the Stazione Zoologica of Naples for the use of their research facilities.

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Summary

1. In *Strongylocentrotus pulcherrimus* eggs, the granules seen in the living egg as orange-colored in white light and dark-red in light filtered through a Wratten No. 49 filter, and the large blackened granules seen in osmium-fixed sections, have been shown to be identical on the basis of size, position and behavior.

2. In unfertilized eggs, these granules are located directly beneath the surface, in which case it is difficult to distinguish them individually.

3. On fertilization, these granules migrate inward from the surface by 1.5 μ, leaving a finely granular zone of the same width at the cell periphery. This zone is called the " extra-granular zone " in the present paper.

4. During the process of segmentation, the equatorial surface of the uncleaved egg with the extra-granular zone and the granular layer forms the cleavage furrow without change in the mutual relations of the component parts except for the fact that the extra-granular zone on the polar side becomes thinner and the granules become more numerous on the sides of the furrow.

5. After the completion of cleavage, the accumulation of granules in the furrow disappears and the extra-granular zone on the polar side regains its pre-cleavage width—i.e., the distribution of the granules around the blastomeres momentarily becomes uniform.

6. The above stage is immediately followed by a phase of formation of new surface along the furrow region in which the extra-granular zone retreats from the sides of the furrow and the granules come into direct contact with the protoplasmic surface film. This arrangement on the new surface is the same as that of the unfertilized egg.

7. Kaolin particles adhering to the cell surface at the head of the cleavage furrow later come to lie at the border between the old surface (provided with the extra-granular zone) and the new surface (which lacks this zone). This is taken as an indication that the new surface is formed from the tip of the furrow, and that the protoplasmic surface film which is carrying the kaolin particles does not slip over the underlying cortex.

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THE BIOLOGICAL BULLETIN

PUBLISHED BY
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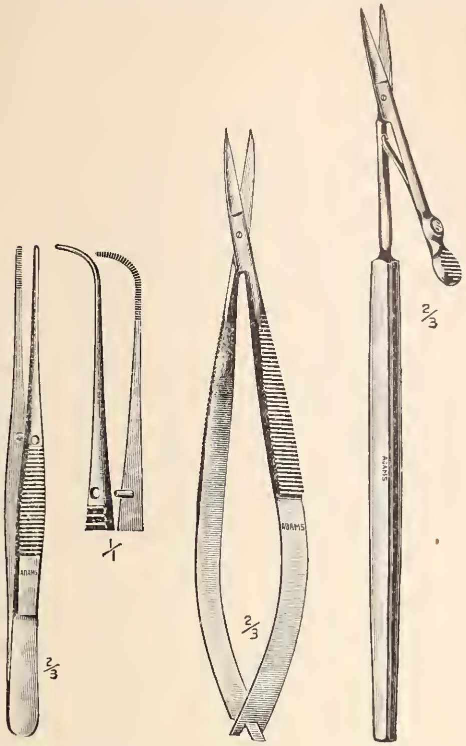

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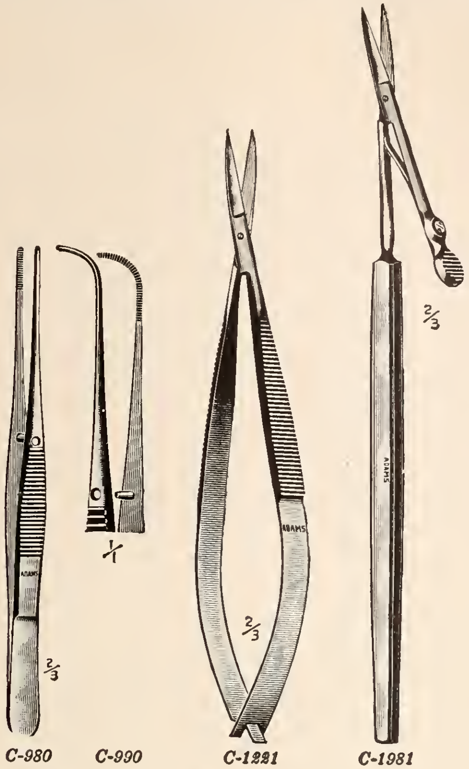

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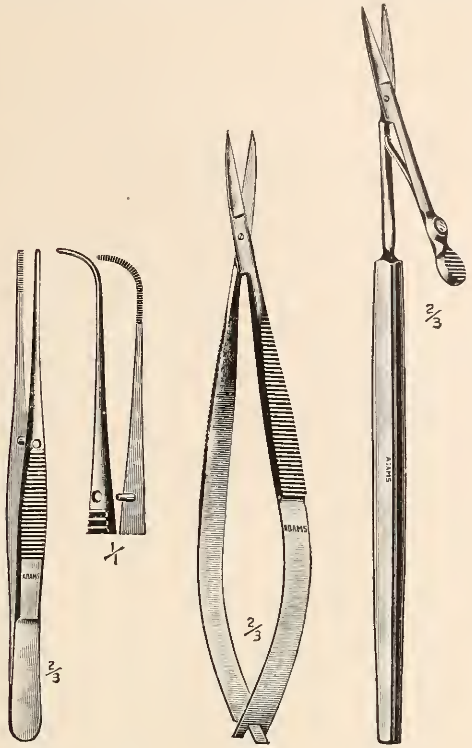

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